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STAIN TECHNOLOGY

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CONTENTS

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LABORATORY HINTS FROM THE LITERATURE

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By H. J. CONN

Published October, 1936

This book, prepared with the collaboration of the Executive Committee of the Biological Stain Commission, is now quite well known as a laboratory guide dealing with the nature and uses of the dyes employed in microscopic work. The third edition is about 50 pages longer than the second, nearly twice as large as the first edition which was published in 1925. The new material consists in large part of the descriptions of 23 additional dyes, most of which have been suggested for biological use within the last five or six years. In addition to this, the statements concerning the stains have been generally revised and brought up to date, frequently with details of new applications. Also a new appendix has been added giving the chemical and biological tests by means of which stains are judged before receiving the Commission certification.

This book (275 pages) treats its subject primarily from the viewpoint of the biological laboratory worker. The chemistry of dyes is discussed, but with the idea of giving information desired by the biologist rather than by the chemist. It discusses about 165 dyes, and gives in detail about 75 of the most important staining formulae and procedures calling for stains. This latter feature makes it a useful laboratory handbook.

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A TECHNIC FOR DIFFERENTIAL STAINING OF NUCLEOLI AND CHROMOSOMES[1]

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ABSTRACT.—A procedure is described which enables a stain to be definitely located in the substance of the nucleolus. Material is fixed in either Navashin or Levitsky; the chromatin is stained by means of the improved Feulgen technic introduced by de Tomasi, and preparations brought thru the washing solutions down to distilled water. From distilled water the material is transferred to a mordant solution, 5% sodium carbonate in water, in which it is left for at least one hour. After mordanting wash well with water then stain for ten minutes in light green solution (90% alcohol, 100 cc, light green SFY, 0.5 g, aniline oil, 2 drops, well shaken); differentiate in alcoholic sodium carbonate solution, (70% alcohol saturated with carbonate); treat with 95% alcohol, absolute alcohol, equal parts xylene and absolute alcohol, clear in pure dry xylene and mount in neutral balsam. Cytoplasm and karyolymph should be quite clear, with magenta chromatin and well defined green nucleoli. The light green does not behave like a simple counterstain as in previous technics but as a definite stain for nucleolar material.

Clear differentiation between chromosome material and the substance of the nucleolus is, at the moment, a matter both of interest and importance in connection with the various theories of the nucleolar functions now being widely discussed. The two types of substance usually associated with nucleoli and distinguished as chromatin and plastin, behave quite differently from the substance of the chromosomes when treated with certain chemical reagents. The simple method of differential staining to be described below was arrived at in the first instance from theoretical considerations of the

[1]The manuscript of this article was received for publication on March 10, 1938.

[2]Grateful acknowledgment is due to Prof. R. Ruggles Gates F. R. S. for the facilities to carry out this work in his laboratory at King's College, University of London.

probable chemical differences between the chromatin of the chromosomes and the substance of the nucleolus. The theory upon which the method is based suggests that altho the nucleoli of different plants and animals may vary somewhat in their response to the treatment, all should be stainable by slight significant variations of the technic.

Certain kinds of nucleoli are said to give a positive reaction with Feulgen's staining technic and also to stain quite readily with the ordinary mordanted nuclear dyes in common use. Ludford (1928) found that both oxyphil and basophil nucleoli in the oöcytes of mollusca gave negative results with the Feulgen test; he makes use of an ordinary counterstain and indicates that the chromosomes may contain other substances in addition to chromatin. The effects of various fixatives on the subsequent behavior of nucleoli towards stains have been observed by quite a number of workers, Sato (1928), Zirkle (1931), Shinke and Shigenaga (1933), Carlson (1936). Van Camp (1924) obtained some degree of differential staining by using Heidenhain's hematoxylin with Congo red and also the rather capricious Ehrlich-Biondi; Marshak (1931) used hematoxylin. Lenoir (1930), using basic fuchsin and malachite green, succeeded in staining the nucleolus; this stain, however, seems to have been fugitive.

There appears to be ample room for a more detailed study of the general behavior of the nucleolus towards fixatives, mordants and dyes. Preliminary considerations of some of the problems involved have led to the working out of the following simple technic for the differential staining of chromosomes and nucleoli. Attention is directed here to the distinction which is to be made between an ordinary general counterstain and a stain that can be definitely located in nucleolar material and cleared away from the rest of the cell. The results obtained up to the present are sufficiently attractive to warrant the publication of an outline of the practical procedure for the benefit of those likely to be interested in problems associated with the nucleolus.

Material may be fixed in either Navashin's, or Levitsky's solutions, preferably cutting down the acetic acid content to a minimum; the chromatin is then stained by means of the Feulgen reaction, using the improved technic introduced by de Tomasi (1936) and incorporating dye prepared according to the method given by Scanlan and Melin (1937), conversion of pararosanilin base (Gurr), C.I. 676, to the acetate, as Sample No. 13. For the subsequent special treatment the following solutions are required:

Sodium carbonate solution, 5% aqueous, (filtered).
70% alcohol saturated with sodium carbonate (filtered).
Solution of light green made up as follows: 90% alcohol, 100 cc.;

light green SF yellowish (Gurr), C.I. 670, 0.5 g.; aniline oil, two drops; shake well and use fairly fresh.

After staining by the Feulgen technic the material is given the following treatment from distilled water:

(1) Mordant for 1 hour in the aq. sodium carbonate solution.
(2) Wash in dist. water (leaving in this for about 30 min.).
(3) Stain for 10 min. or longer in the light green solution.
(4) Rinse with alc. sodium carbonate solution.
(5) Transfer to 95% alcohol and leave for at least 10 min.
(6) Examine under the microscope, if the cytoplasm is not free from green, rinse again in the alc. carbonate and transfer to 95% alcohol. The art of judging the correct differentiation can soon be acquired.
(7) Leave in abs. alcohol 10 min. or longer.
(8) Equal parts xylene and absolute alcohol, 10 min.
(9) Clear in pure xylene; (differential staining is more clearly defined after xylene).
(10) Mount in neutral balsam.

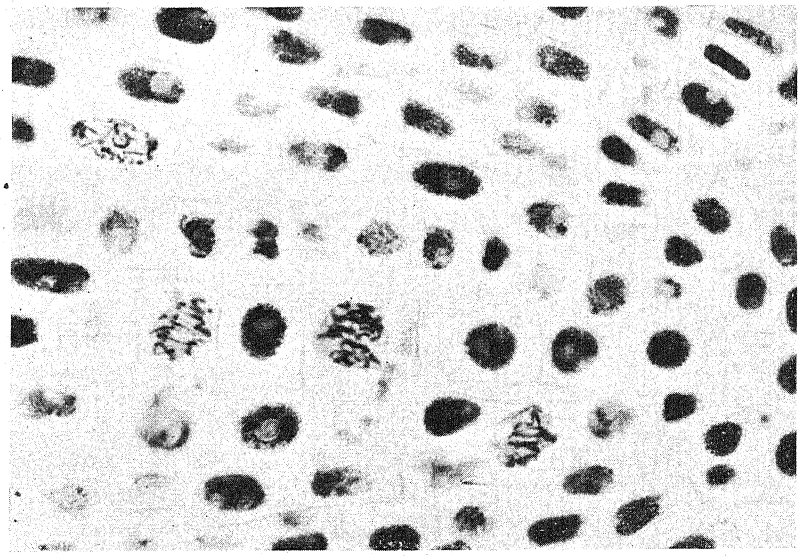
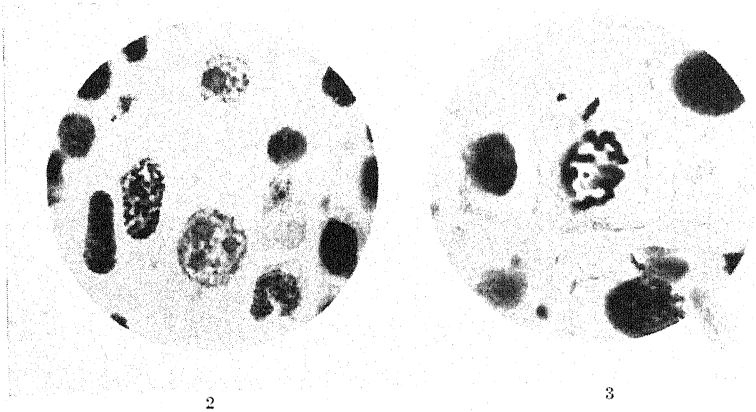


FIG. 1. Longitudinal section of the root tip of *Scilla nutans*, under low power to show nuclei in various stages. Chromatin has been stained magenta by means of the Feulgen reaction and nucleoli green by the new differential staining; cytoplasm and karyolymph are unstained.

Nucleoli are stained from light to dark green and are clearly demarked from the magenta chromosomes and colorless karyolymph. The characteristic color of the chromosomes, due to the Feulgen reaction, is neither masked nor debased by the green, which by careful

differentiation may be washed out from everything but the nucleolar material.

Normal eyes can distinguish very clearly between the colors red and green, but the difference, so easy to see, is extremely difficult to render in any marked degree of contrast with the available photographic materials. The accompanying figures show definite differences of tone between nucleoli and chromosomes, but they do not give an adequate impression of the brilliancy of the preparations or the sharpness of the color distinction. Fig. 1 shows a longitudinal section of Scilla root tip under low power and gives a general idea of



2 3

FIG. 2. Resting stages in root tip of *Scilla nutans* showing stained nucleoli with clear karyolymph. Chromatin threads have responded to the Feulgen reaction.

FIG. 3. Prophase in root tip of *Scilla nutans*. Chromatin deeply stained by the Feulgen. The differential staining of the nucleolus and satellite is clearly seen.

the effectiveness of the stain; Fig. 2 indicates how clearly the nucleoli are picked out in the resting stage and Fig. 3 shows a prophase in which may be plainly seen a chromosome with a satellite attached to the nucleolus.

The facilities which the technic offers for the critical study of relationships between nucleoli and chromosomes will be at once apparent, while there is every possibility that the method may also help in the elucidation of various interesting problems connected with fertilization.

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CLEARING SPECIMENS FOR THE DEMONSTRATION OF BONE

R. W. CUMLEY, J. F. CROW, and A. B. GRIFFEN, *Department of Zoology, University of Texas, Austin, Texas*

ABSTRACT.—A brief review is given of some of the older technics of clearing and staining various types of animals, in such a way that the bones are clearly visible in the surrounding tissues. The authors have modified some of these earlier practices. Particularly good results were obtained by combining the Schultze technic with that of Lundvall. Embryos are fixed in 95% alcohol, treated with 1% KOH, stained in 1:10,000 alizarin Red S, cleared in gylcerin, dehydrated with alcohol and further cleared in toluol, toluol saturated with naphthalene, and anise oil saturated with naphthalene. Details of the technic are presented in the text.

Several technics have been suggested for rendering opaque objects transparent. In practically all cases the methods involve a depigmentation and dehydration of the tissue. One of the most successful applications of such technics is in the demonstration of skeletal parts, since the bones can be observed without dissection. Particularly in the field of embryology are such preparations useful, since they serve to present material for the study of ossification. The purpose of this paper is not so much to present new methods but rather to review some of the more successful older technics and to suggest some modifications which have been found to improve the quality of the results.

One of the best known methods is that of Spalteholz as reviewed by Sabin (see Guyer, 1930). In this method the embryo is bleached in hydrogen peroxide and cleared in benzol and synthetic oil of wintergreen. Some of the disadvantages of the technic are: (1) hydrogen peroxide causes the tissue to become filled with bubbles which are often difficult to remove, (2) hydrogen peroxide is not as efficient as a bleaching agent as are some other compounds, (3) the tissue tends to turn brown with age, and (4) oil of wintergreen causes the tissue to shrink. We have found that Batson's method of removing bubbles with a vacuum pump is desirable when the use of hydrogen peroxide cannot be avoided. Care must be taken, however, as the pressure within the tissues, created by evacuation of the surrounding area, often destroys or displaces the internal organs. Hydrogen peroxide is particularly useful in bleaching large insects whose chitinous cover-

ings are not disrupted by the bubbles (Stapp and Cumley, 1936). Sabin has modified the Spalteholz technic by fixing in Carnoy's fluid. This not only bleaches the tissue better than hydrogen peroxide, but also slightly swells the tissue and thereby counteracts the shrinking effect of the oil of wintergreen.

Another group of technics which are widely used are the methods of Schultze (1897) and Mall (1906) and modifications of these. In all of these procedures potassium hydroxide is used for bleaching and glycerin for clearing. Dawson has devised a method of staining bones with alizarin while clearing the soft tissues by Mall's method (Dawson, 1926). The disadvantage of using KOH is in its strong tendency to macerate tissue, particularly in embryonic material. Nevertheless, by carefully watching for maceration the danger can be avoided. The glycerin clearing method does not seem to produce the great transparency that is apparent in preparations cleared in the various oils. We have, however, obtained good results in using KOH and glycerin for clearing small fish, particularly if they are thin. Fish are not as easily macerated as embryos and hence are not likely to be injured by the strong alkaline solution. The Hollister (1934) technic for clearing fish is as follows:

1. Fix for several days in 70% alcohol.
2. Bath in dist. water for a few minutes.
3. Place in 1–4% KOH until the bones become apparent in the clear surrounding tissue.
4. Stain from 6–24 hr. or more, depending on the size of the specimen, in alizarin dye solution prepared as follows:

Alizarin, sat. solution in glacial acetic acid, 50%	5 cc.
Glycerin	10 cc.
Chloral hydrate crystals, 1% sol.	60 cc.

Place 1 cc. of the above solution in 1000 cc. of 1% KOH.

5. KOH, 1–20% without glycerin.
6. KOH, 1–4% with 40% glycerin.
7. Gradually increase the amounts of glycerin. The time between changes varies with the individual.
8. Store in glycerin.

Hollister recommends the use of ultra-violet light in the clearing process. This has the effect of bleaching and hastening the clearing. Two modifications of this technic may be suggested. First, we have found that by fixing in 95% alcohol instead of in 70% the chances of maceration are decreased. Second, alizarin red S dissolved in 1% KOH or in distilled water, in the proportion of 1 part of dye to 10,000 parts of the solvent, produces results quite as good as the more elaborate dye solution of Hollister. A photograph of a fish cleared by this modified Hollister method is shown.

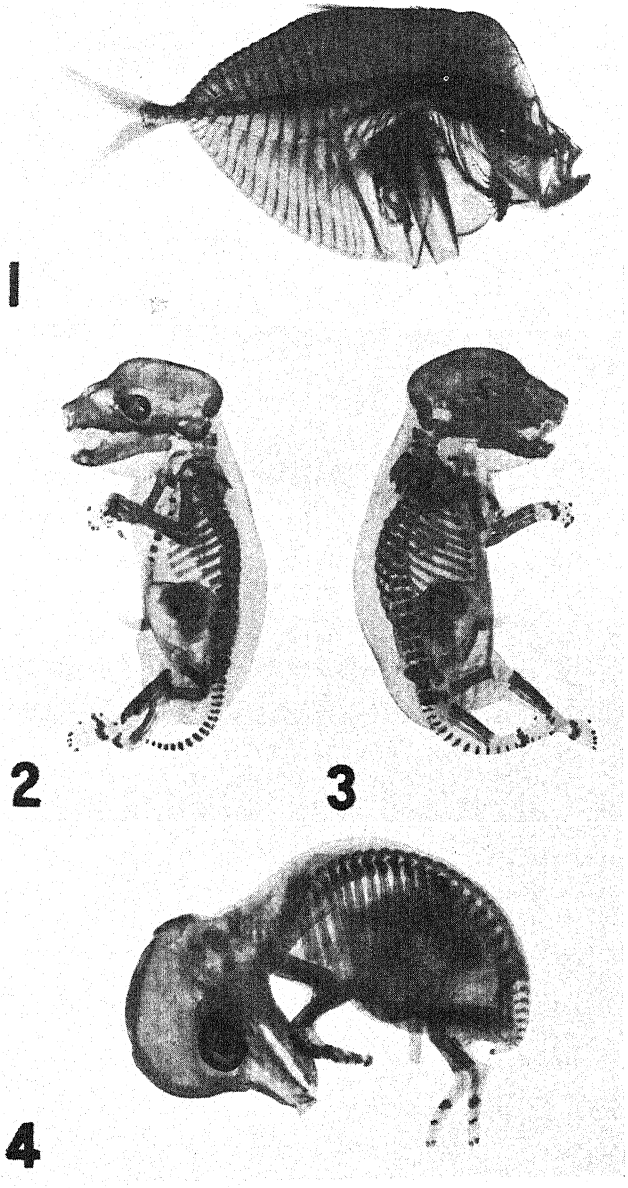

FIG. 1. FLATFISH

FIG. 2. SQUIRREL EMBRYO

FIG. 3. SQUIRREL EMBRYO

FIG. 4. PIG EMBRYO

All approximately natural size

For the demonstration of bony skeletons of mammalian embryos we have had the most success with a combination, with some modifications, of the KOH clearing methods and that of Lundvall (1927). Bleaching in KOH seems to produce better results than the oxalic acid which Lundvall recommends, and the danger of maceration is reduced by the addition of alcohol to the KOH when necessary. Toluol was substituted for benzene as a clearing agent because, while it clears equally well, it does not render the tissue so brittle (Galigher, 1934). The technic with which we have had the best results is as follows:

1. Fix for several days in 95% alcohol.

2. Wash in water a few hours.

3. Place in 1% KOH until the tissue surrounding the bones becomes clear and jelly-like. This requires from a few hours to a week or more, depending on the size and type of the embryo and the length of time fixed in 95% alcohol.

4. Stain in a solution of alizarin red S (alizarin sodium sulfonate) made up in the proportions of one part of dye to 10,000 parts of distilled water to which a few drops of dilute KOH have been added to make the solution basic. We have used the Coleman and Bell, Hartman-Leddon, and Eimer and Amend products. All were good, but none had Commission certification numbers on them. The embryo should remain in this solution for 3–12 hours.

5. Replace the dye solution with 1% KOH which contains about 10% glycerin. Gradually add equal amounts of glycerin and alcohol until the KOH has been completely replaced. During steps 3 to 5, if the embryo shows signs of maceration, gradually add alcohol until the concentration is 80% or 90%, and allow to harden a few days before resuming the clearing process. The embryo by this time should be completely destained except for the bone, which is red. Placing the embryo in sunlight hastens the decolorizing process.

6. Gradually add 95% alcohol until all the glycerin has been replaced. The changes must be gradual or shrinkage results.

7. Gradually replace the 95% alcohol with absolute alcohol. Change the solution several times to insure complete dehydration.

8. Replace the alcohol gradually with toluol. Change the toluol several times.

9. Change the embryo to toluol which has been saturated with naphthalene.

10. Replace this solution with anise oil saturated with naphthalene, and store in this solution.

Photographs of three embryos cleared by this method are shown. In these cases the viscera of the embryos were not removed, but in large embryos it may be necessary that this be done to obtain complete transparency. In large human embryos it has been suggested that the brain also be removed.

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THE USE OF THE FEULGEN TECHNIC WITH CERTAIN PLANT MATERIALS

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ABSTRACT.—**The Feulgen technic as modified by Heitz promises to become an extremely useful tool in the solution of certain cytological problems. A procedure is outlined for using this technic with root tip smears, and smears of plant microspores. The chief improvement suggested over previous methods is that the material be mounted in euparal, after immersion in 95% alcohol. The technic is of value in the study of chromosome fragmentation, chromatid coiling, centromeres, etc., in both somatic tissue and in microspores.**

INTRODUCTION

The Feulgen technic, or so-called "nucleal reaction", has considerable promise as a cytological stain for both plant and animal materials. According to de Tomasi (1936) the stain was originally developed by Feulgen and Rossenbeck as a microchemical test. It depends upon the use of a reduced or colorless form of basic fuchsin which, upon contact with an aldehyde, develops a specific purple color.

Successful application of the Feulgen technic depends chiefly upon securing a suitable form of basic fuchsin. Conn (1937) states that the solution of this problem is at hand. Some intensive research into the chemistry and staining properties of this dye has indicated procedures that will give a satisfactory product (see Scanlan and Melin, 1937).

The writer has obtained basic fuchsin from commercial sources, manufactured specifically for use with the Feulgen reaction. Such products have proved to be entirely satisfactory and reliable.

The directions of de Tomasi (1936) should be followed very closely in making up the stain. It must be kept in mind that the reaction is contingent upon the ability of basic fuchsin to decolorize in the presence of sulfite, consequently the stain should not be used unless it has been completely decolorized.

The work of Margolena (1932) indicates that the Feulgen reaction can be used with a wide variety of botanical materials in cytological and histological studies. Heitz (1936) has devised a method of applying the Feulgen technic to root tip smears. He omits the sulfite

washes after staining, and substitutes 45% acetic acid. In acetic acid of this strength the stain does not remain in the cytoplasm. This eliminates the necessity for passing the material thru successive sulfite washes and a counterstain.

Klingstedt (1937) has had good success with grasshopper testes following the same general procedure as that used by Heitz. The principal difficulty has been to find a satisfactory method of making the slides permanent.

The writer has modified and extended the procedure used by Heitz, (1) by introducing methods of making the smears permanent; (2) by applying the technic to smear preparations of plant microspores.

With the Feulgen technic, the root tip smear method has been used with considerable success in obtaining chromosome counts where the

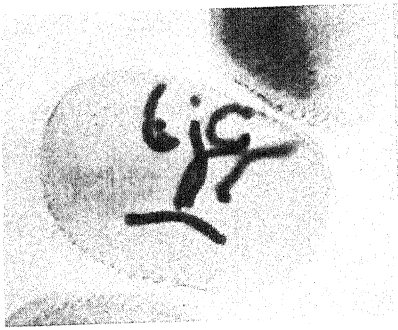

FIG. 1. Microspore of *Tradescantia paludosa* Anderson and Woodson. This photomicrograph shows the haploid number of 6 chromosomes + 4 fragments.

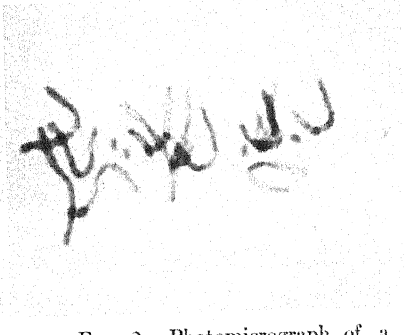

FIG. 2. Photomicrograph of a root tip cell of the same species, showing the diploid number of 12 chromosomes + 6 fragments. × 1200.

Thanks are due to Prof. Karl Sax, Biological Laboratories, Harvard University, for these photomicrographs.

material has small chromosomes as one finds in such genera as *Lactuca*, *Cichorium*, *Cucumis*, and certain species of *Asparagus*. It can also be employed with profit in studies of chromosome fragmentation, chromatid coiling, location of centromeres, secondary constrictions, and satellites where the material has large chromosomes (*Tradescantia*, *Lilium*, etc.; see Fig. 2).

As La Cour (1937) has stated, root tip smears are open to the criticism that it is often difficult to find a sufficient number of plates in polar view to make satisfactory counts of the chromosomes. Where the material has fairly small chromosomes, however, and where they are not too numerous as in most species of *Lactuca*, or where the chromosome number is small as in species of *Tradescantia*, root

tip smears making use of the Feulgen technic seem to have promise. Feulgen preparations of microspores of *Tradescantia*, *Aloe* and *Lilium* were found useful in the study of fragmentation, centromeres, etc., (see Fig. 1). The prophases of such material are extremely well fixed.

Procedure

Fixation: In the writer's experience it is quite important that a fixative be employed which contains a minimum of acetic acid. Benda's fluid (low acetic) made up according to the directions of La Cour is perhaps one of the best fixatives for use with the Feulgen reaction. Good results have been obtained by using Benda's fluid without acetic acid. Navashin's solutions can be used, but they do not give as uniformly good results as do fixatives containing osmic acid.

The time of fixation varies considerably with different types of material. For root tips, 15–20 minutes may be sufficient. For entire anthers with the enclosed microspores, 30–45 minutes may be required.

Hydrolysis: This operation takes place in *N* HCl at a temperature between 50–60° C. The length of time allowed for hydrolysis is one of the critical points of the technic. In general, it is governed by the length of the period of fixation, i.e., if fixation lasts 30 minutes, hydrolysis should extend over the same period.

Staining: After hydrolysis, the material is placed in the stain from 15–20 minutes, and then transferred directly to a dish containing 45% acetic acid (10–15 minutes or longer). The writer has found that some materials overstain easily, making it difficult or impossible to clear the stain out of the cytoplasm, and for this reason the material should not remain in the staining solution too long.

Mounting: After immersion in 45% acetic acid the material is transferred to a slide and placed in a drop of acetic acid of the same strength. Root tips are dissected into thin strips of tissue, if possible, only a few cells in thickness. Anthers are opened by an incision at one end, and the contents pressed out. The remnants of anther walls and other debris are removed. The cover slip is placed over the material, and pressure applied until it has reached the desired thickness. Considerable pressure can be applied to root tip smears without damage, but with microspores care should be taken so that individual cells are left intact. The slide should be heated to nearly the boiling point at least three times, some pressure being applied each time to the cover slip. This operation insures the material adhering either to the slide or the cover slip.

Making permanent: The cover slip is floated off in a mixture of equal parts of absolute alcohol and glacial acetic acid. Both cover slip and slide are transferred to 95% alcohol from this mixture. They are permitted to remain in this solution for at least 15 minutes and are mounted in euparal. The latter step must be carried out in a room where the relative humidity is low, and care should be taken to avoid breathing on the slide, otherwise there is danger of it becoming cloudy or white. The writer has slides prepared according to the directions outlined above which are over one year old, and show no signs of deterioration.

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MODIFICATIONS OF LEBOWICH'S SOAP-WAX TECHNIC

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ABSTRACT.—Lebowich's technic is outlined for simultaneous dehydration and infiltration of tissues by a medium composed of stearic acid, 56° C. paraffin, diethylene glycol, and monoethanolamine. The prices and places where these materials may be purchased are given.

Tissue for sectioning is placed in acetone, C.P., for 1 hour, then put directly into the soap-wax medium at 60° C. under reduced pressure, and finally embedded in new soap-wax.

Modifications include a simplification of the apparatus used by Lebowich. A preserving jar fitted with a rubber stopper serves as a vacuum chamber, and use of an aspirator accomplishes the reduction of pressure. With invertebrate embryos up to 1000 μ diameter no reduction of pressure is needed. Embryos are fixed, washed, placed in acetone, infiltrated in soap-wax, and embedded.

By this soap-wax method the alcohols, xylene, and overnight drying of affixed ribbons are eliminated. Tissue may be fixed, sectioned, stained, and permanently mounted within 6 to 8 hours.

In 1936 Lebowich[1] published a technic using soap-wax as a medium for simultaneous dehydration and infiltration of tissues. This technic with modifications has offered several advantages for sectioning molluscan embryos, and I believe others will find its application valuable.

Since the journal in which Lebowich published is one not usually read by biologists, it seems advisable to state Lebowich's general method while considering modifications of it.

The soap-wax medium is made from the following:

100 g. of XXX saponified stearic acid for research purposes, obtainable from Harkness and Cowing Co., Cincinnati, Ohio, at about $1 per pound.

400 g. of 56° C. paraffin, C.P.

7.5 g. of diethylene glycol, C.P., obtainable from City Chemical Corp., 132 West 22nd Street, New York City, at 50c. per 100 g.

7.5 g. of monoethanolamine, C.P., obtainable from Carbide and Carbon Chemicals Corp., South Charleston, West Virginia, at $1 per pound.

[1]Lebowich, R. J. 1936. Rapid, reliable and simple technic for the simultaneous dehydration and infiltration of human tissues. Arch. Path., 22, 782–805.

The stearic acid and paraffin are each melted and then stirred together at 85° C. at high speed in a glass container, preferably by an electric mixer, for about 5 minutes. The amounts of diethylene glycol and monoethanolamine are added, and the mixture is stirred for an additional 30 minutes at high speed and at 85° C. Allow the heat to drive off the bubbles after stirring. When cooled, the medium resembles ivory soap in color and texture. Many of the chemical and physical properties of the soap-wax solution are published in Lebowich's paper.

The infiltrating apparatus used includes a short wide-mouthed jar fitted with a No. 13 rubber stopper. Thru the rubber stopper a glass tube is fitted tightly, and 3 or 4 feet of vacuum tubing is attached to the glass tube. During the process of embedding, the jar is placed in an oven to obtain the correct temperature. The vacuum tubing leads out of the oven to an aspirator attached to a faucet. By means of a glass Y-tube a vacuum gauge is attached along the line to denote the vacuum created by running the water thru the aspirator. Lebowich's apparatus is much more complicated and expensive, as he uses a motor driven vacuum pump and special vacuum chamber. The simpler and less expensive articles do just as well.

To embed tissue:

Fix in any ordinary fixative. If corrosive sublimate is an ingredient, the tissue must be washed in water or a low percentage of alcohol before placing the tissue in acetone.

Transfer to acetone, C. P. for 1 hour with a change of solution after 30 minutes. With molluscan embryos 600 to 1000 μ in diameter 30 to 40 minutes have been found to be sufficient with no change of fluid. The purpose of the acetone is to extract fats which are not penetrable by the soap-wax.

Place the tissue in a dish containing melted soap-wax. Put the dish into the wide-mouthed jar and plug the latter firmly with the rubber stopper. Set the vacuum chamber in an oven at 58° to 62° C.

Reduce the pressure to ½ atmosphere (400 mm. or 15 inches) with the aspirator. Take about 15 minutes for this reduction of pressure by twisting the faucet every 2 or 3 minutes until the desired reduction is reached.

Clamp the vacuum hose shut and allow the tissue to stand for 40 to 45 minutes. The melting point of the soap-wax is 49.3° C., but better infiltration is obtained if the temperature is kept at 56° to 62° C.

Unfasten the vacuum hose from the aspirator and by loosening the clamp allow a gradual return to normal atmospheric pressure in about 15 or 20 minutes.

Transfer the tissue to a new dish containing melted soap-wax.

Orient the tissue with a cat's whisker or pig bristle stuck into the end of a glass tube by sealing wax, and allow the mixture to harden.

There is no need for immersion in water as the soap-wax solution is non-crystalline.

Section in the usual way. The ribbon sections better at 5 than at 10 μ.

With small objects such as the invertebrate embryos mentioned, reduction of pressure in the vacuum chamber is not necessary.

Tissues stored in alcohol for a considerable length of time may go directly into the soap-wax solution from the alcohol, omitting the acetone.

If yolk offers undue difficulties, it will section satisfactorily if soaked 8 to 12 hours in a solution of 8 g. of phenol crystals in 100 cc. of alcohol of any percentage from 35 to 80. The block should be chilled before cutting.

To affix sections to slides when serial sections are not desired, Lebowich's method works well.

Float the sections on a bath of 2.5% aq. solution of gum arabic at 45° to 48° C.

After the sections have stretched sufficiently transfer them for about 10 seconds to a water bath to remove the excess soap-wax. This may be done by using a clean glass slide.

After 10 seconds dip the sections onto the proper position on a slide prepared as indicated below. A longer time on the water bath may cause the sections to break into bits. Wipe away the excess fluid.

Dry on a warming plate at 45° to 50° C. for 1 hour. The sections are then ready to stain.

For affixing serial sections the following procedure has been devised:

Place a few drops of unheated 2.5% solution of gum arabic on a slide prepared as indicated below and transfer the ribbons in proper order onto this fluid.

Place the slide on a warming plate at 45° to 50° C. and stretch the ribbons as is normally done with paraffin technic.

Drain the gum arabic solution from the slide by holding a cloth around one end of the slide and tilting the slide gently.

When most of the gum arabic solution has been absorbed by the cloth, drop 15 to 20 drops of dist. water heated to 45° C. at the upper end of the tilted slide. Allow the water to wash under the ribbons, carrying away the excess gum arabic solution. Drain off as much of the water as possible around the sections and then place the slide on a warming plate at 45° to 50° C. for 1 hour. The slide is then ready for staining. This procedure requires a little skill to prevent the sections from adhering to the cloth when the slide is tilted, but it is easily done after a trial or two.

Preparing slides with adhesive requires a gelatin solution, as the albumen method does not work well for soap-wax ribbons. The gelatin solution is prepared as follows:

Allow 0.25 g. of a good grade of granular gelatin (Lebowich recommends No. 850,669 granulated gelatin by Milligan and Higgins Corp.) to swell in 10 cc. of dist. water in an ice box for 30 minutes. Lebowich uses 0.5 g. The amount depends on the gram strength of the gelatin and must be determined by experiment.

Add 89 cc. of dist. water and 1 cc. of hydrogen peroxide.

Gently heat until the gelatin goes into solution.

Slides are prepared as follows:

Clean slides by immersion in a solution of equal parts of 95% alcohol and CCl_4. Allow the slides to evaporate to dryness.

Dip the cleaned slides into the gelatin solution.

Drain them so as to leave an even coating of gelatin. Dry.

Dip gelatin covered slides into a 5% solution of neutral formalin.

Dry and store for use.

The procedure for staining the sections on the slides is as follows:

Place slides with the ribbons affixed into CCl_4, C.P., for 10 minutes to dissolve the soap-wax.

Transfer to 95% alcohol, 3 to 5 minutes.

Two changes of tap water for a few minutes each.

Stain.

95% alcohol.

Two changes of 100%.

1 part xylene to 1 part 100% alcohol.

Mount in euparol.

The greatest difficulty with the technic is in the time and effort to obtain the materials needed. They are, however, of low cost. Once the soap-wax is made, the technic runs smoothly and has the following advantages over the ordinary paraffin methods:

Dehydration and infiltration are accomplished simultaneously.

The alcohols, or similarly used intermediate fluids, are omitted; and with minute objects the less changing of fluid the better.

Soap-wax will not crystallize when cooling.

Where difficulty in complete dehydration is encountered by alcohol or dioxan, such as is the case at marine stations, the soap-wax works remarkably well. Thus invertebrate embryos may be embedded directly instead of being left in fixing fluid where they often adhere to one another or collect debris before subsequent handling.

Soap-wax gives an excellent ribbon at 5 μ, in fact, it sections better at 5 than at 10 μ.

The technic is rapid and the work from living tissue to finished slide may be accomplished in 6 hours or less, if the materials needed are at hand. No overnight drying of slides is necessary.

STAINING PARAFFIN SECTIONS WITH PROTARGOL

3. THE OPTIMUM pH FOR REDUCTION. 4. A TWO-HOUR STAINING METHOD[1]

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ABSTRACT.—Further work on conditions affecting the reduction of paraffin sections impregnated with protargol showed that the optimum pH for sulfite-amidol mixtures was between 6.5 and 7.5. A staining method which requires about two hours to complete consists of the following steps: (1) One hour impregnation at 60° C. in 10% $AgNO_3$. (2) Wash in distilled water 3 changes of 30 sec. each. (3) Put into protargol (Winthrop Chem. Co., New York, N. Y.) 0.2% aq. for another hour at room temperature. (4) Rinse 2 sec. (5) Reduce one to two min. in amidol 0.2 g., Na_2SO_3 8 g., $NaHSO_3$ 1 g., and water 100 cc. (6) Wash thoroly. (7) Tone with 0.1% gold chloride. (8) Wash. (9) Reduce with a 0.5% aq. soln. of amidol (no sulfite). (10) Wash, dehydrate and cover. The method stains neurofibrillae and unmyelinated fibers and has worked well on most tissues of vertebrates. The stain follows acid alcoholic fixation.

The use of protargol as a nerve fiber stain was discussed in a previous paper (Davenport and Kline, 1938). An improved fixative which consisted of trichloracetic acid, 10 g.; formic acid, 5 cc.; dissolved in a mixture of normal propyl alcohol and normal butyl alcohol (or of normal propyl alcohol alone) to make a final volume of 100 cc. was suggested as a modification of Hofker's fixative. The use of a second reduction after gold-toning, as a means of intensification of the stain, was discussed also. Subsequent experiments have led to the development of a staining method for axis cylinders which requires about two hours to complete. This gives, as a rule, better differentiated preparations than the previous procedure, which required about two days.

PART 3. THE OPTIMUM pH FOR REDUCTION

Since there are two reductions required in the staining process, the following comments apply to the first reduction, in which the protargol is reduced with an amidol-sulfite mixture.

[1]Contribution No. 280 from the Department of Anatomy, Northwestern University Medical School.

Paraffin sections of nervous tissue (dorsal roots, sciatic nerves and sympathetics of cats) were impregnated with 0.5% protargol for two days at room temperature, rinsed two or three seconds in distilled water and reduced with mixtures of 0.5% amidol in 5% sodium sulfite plus either acetic acid or sodium hydroxide to give a range in pH between 4 and 10. We had hoped that better differentiation of neural elements might be obtained by the acidification of the reducing solution. The advantages were slight, however, and were offset by the tendency for precipitate to form on the slide when the acidity of the reducer was near the isoelectric point of the protargol (about pH 4). The optimum for most material seemed to lie between pH 6.5 and 7.5 and was obtained by adding 0.5 cc. of glacial acetic acid to each 100 cc. of the amidol-sulfite mixture.

As mentioned in the preceding paper, differentiation was improved more effectively by reducing the concentration of amidol to 0.1% or 0.2% and increasing the sulfite concentration to 8 or 10%. This latter finding has been used in the rapid method to be described.

Part 4. A Two-hour Staining Method

The following method has some points in common with Foot's silver nitrate method (1932) in that 10% silver nitrate has been used for impregnation at a temperature above room temperature; a rather dilute solution of reducing agent used for the silver reduction; and a second reducing solution applied after toning. We have introduced a dilute protargol solution as a second impregnating medium, and found that its advantage lies in permitting the use of a buffered amidol-sulfite to secure good differentiation of nerve fibers, completeness of staining, and a minimum of trouble with precipitate.

The observation, described in the earlier paper, that nervous tissue fixed in Hofker's mixture or a modification thereof was readily impregnated by aqueous silver nitrate led to the present use of a combination of impregnations of silver nitrate and protargol in sequence. This procedure has improved the differentiation of nerve cells and fibers from connective tissue in all preparations and possesses the additional advantage of greatly shortening the time required for impregnation. An arbitrary procedure will be given, followed by comments on the various steps.

1. Fixation. Fix small nerves about two hours, large tissue blocks overnight, in the following mixture:

Pure formic acid (about 85% conc.)	5 cc.
Trichloracetic acid	5 g.
Normal propyl alcohol	25 cc.
Normal butyl alcohol	65 cc.

(To avoid esterification, mix just before using.)

2. Transfer to 95% ethyl alcohol then thru several grades of ethyl to water, allowing one to two hours for each change; dehydrate and embed in paraffin in the usual manner. Section and mount.

3. Remove the paraffin and descend thru graded alcohol to water.

4. Impregnate for about one hour in 10% aqueous silver nitrate (Merck's C.P. used) in a paraffin oven at 58 to 62° C.

5. Rinse thru three changes of distilled water, allowing the slides to remain about 30 sec. in each change.

6. Impregnate for an hour at room temperature in 0.2% protargol (Winthrop Chemical Company, New York City).

7. Rinse quickly (2 sec.) in distilled water and reduce in an amidol-sulfite solution made as follows:

Stock Solution A:	Sodium sulfite	10 g.
	Distilled water	90 cc.
Stock Solution B:	Sodium (or potassium) bisulfite	5 g.
	Amidol	1 g.
	Distilled water	95 cc.

For use mix 1 part of Sol. B with 5 parts of Sol. A. Reduction is complete in about one minute.

8. Wash in running tap water for several minutes, and transfer to one change of distilled water. (*Caution:* Do not put slides back to back as this will prevent the complete removal of the reducing agent.)

9. Tone for several minutes, until all the yellow color of the silver stain is replaced by gray, in a 0.1% aqueous solution of gold chloride.

10. Wash as in step 8.

11. Reduce with a 0.5 to 1.0% aqueous solution of amidol, preferably by dropping the solution on the slide. Sulfite must not be added. Reduction is complete in a few seconds.

12. Wash, dehydrate and cover.

Comments on Individual Steps of the Method

1. *Fixation.* Several variations in acid-alcohol fixatives have been tried and the experiments indicate that the stain will follow almost any combination of formic, acetic, or trichloracetic (or combinations of these) in either methyl, ethyl or propyl alcohol. The addition of formalin up to 2 or 3% in the solutions is optional, and, in a few instances, such an addition appeared to improve fixation and staining slightly. A mixture of 5% formic plus 5% trichloracetic acids in water, instead of the propyl-butyl alcohol mixture recommended, gave a good stain on a dorsal root. The trichloracetic acid concentration of 10%, previously recommended, appears to be stronger than necessary. After 50% pyridin fixation of spinal cord of cat,

many synaptic end-bulbs (*boutons termineaux*) were stained, but it appeared doubtful that the staining of these structures was complete. Aqueous 10% formalin, formalin-acetic, and Bouin's fluid have always given results inferior to fixatives based on a solution of trichloracetic acid.

2 and 3. These steps are ordinary routine and were not subjected to experiment.

4. The time, temperature, and concentration of the silver nitrate solution were found to be uncritical but interrelated. Impregnation for several days at 27° C. with a 2% solution gave results similar to those obtained at 60° with a 10% solution in 1 hour. Two hours in 10% silver nitrate at 60° followed by 20 hours at 27° did not cause overimpregnation.

Twenty hours at 60° C. caused excessive staining of connective tissue. From experiments with time intervals shorter than one hour, it appeared that 45 minutes (which included the warming of the solution from room temperature to 60° in the oven) was about the minimum. Solutions of silver nitrate stronger than 10% were not tried. Repeated use of the solutions was found to be practicable since those of 10% strength were used five or six times without apparent impairment of the staining reaction. Presumably they will continue to function properly as long as they remain clear.

5. Rinsing after treatment with silver nitrate was varied from a few seconds to three minutes. The best final impregnations seemed to occur when all excess silver nitrate was removed; hence a total time between one and two minutes has been recommended. The wash water must be distilled water and it should cover the slides completely. It is changed by pouring off and adding fresh water rather than transferring the slides from one container to the next.

6. We have attempted to secure staining by reducing immediately after impregnation with silver nitrate and subsequent rinsing, but this procedure was unsuccessful. Therefore, the second impregnation seems essential. Attempts were made to secure staining by using protargol alone at 60° for two hours, but these failed also, possibly because of too low concentration of the silver ion. After the original treatment with strong silver nitrate solution, however, neither the time of treatment with protargol solution nor its concentration is critical. We have tried varying the time of the protargol impregnation from a few minutes to an overnight period, and believe that at least 30 minutes at room temperature is necessary. Concentrations of 0.1, 0.2, and 0.5% protargol were used, but 0.2% seemed to be near optimum. Material left in the protargol more than one hour showed

no change of staining. We have preferred to use the protargol solutions only once altho they will function for several stainings.

7. Rinsing after the protargol impregnation was found to be optional. Neither of the reducing solutions keep indefinitely if exposed to air, but Sol. A is stable in stoppered bottles. We have not attempted to keep any amidol solutions for more than a few days, since they oxidize rather readily. The solution of amidol in bisulfite provides a buffered solution near neutrality when mixed with sulfite in proportions of 1:10 to 1:4. Differentiation of nervous elements was found to be better when dilutions of one part of Sol. B to 10 of Sol. A were used, but for some material the stain may be too pale. A solution of 0.2% amidol in 8 to 10% sodium sulfite may be used if desired with efficacy about equal to the sulfite-bisulfite mixture.

8. The reducing solution should be completely removed by thoro washing, otherwise rapid deterioration of the gold-toning solution occurs.

9. The gold-toning process is routine except that no hypo solution is used.

10. The washing at this stage merely removes the excess gold chloride solution. If omitted, the gold forms a fine precipitate on the slide during the second reduction, step 11.

11. Some preparations may stain with sufficient intensity to give what is wanted without reduction at this stage, but the stain is usually too pale, especially if 0.2% protargol is used for the final impregnation. Over-reduction does not occur if the excess gold chloride has been removed in the preceding step.

12. This step is routine.

Discussion

The foregoing method, with variations on the various steps, has been tried on spinal nerve roots, sympathetics, peripheral trunks, and a few specimens of the central nervous system of vertebrates. The following types were represented, but most of the material was from the cat: man, monkey, guinea pig, rat, cat, bull frog, and several species of fish. The general degree of success indicates that it has a rather wide range of application, altho, of course, it is not expected to cover all needs of a nerve stain. It has been less successful on large trunks of peripheral nerves than elsewhere, since the difficulty of obtaining perfect fixation seems to be the limiting factor rather than any inherent fault of the staining method.

This method has been checked with the two-day protargol method, previously described, by the direct comparison of slides made from

the same block of tissue and by fiber counts. Dorsal roots of cat, rat and monkey were cut serially and the numbers of fibers in the root found to agree, within experimental error, with the number of cells in the ganglion.

Cat, 4th cervical:	17,700 cells;	16,900 fibers
Rat, 13th thoracic:	5,500 "	5,600 "
Monkey, 1st sacral:	21,400 "	19,300 "

The unmyelinated fibers of the above nerve roots are extremely fine, and the test indicates that all, or nearly all, of the fibers were stained. Direct comparison of counts on three small fascicles of the cervical sympathetic nerves of a guinea pig, stained by both the short and long processes, gave the following:

	Number of fibers	
	2-day method;	*2-hour method*
Fascicle 1.	184	180
2.	644	668
3.	547	587

In this instance, also, the unmyelinated fibers were very fine and the agreement between the two methods gives a strong suggestion of complete staining.

In observations on other preparations stained by both methods it would seem that the 2-hour staining method is more likely to yield higher counts of nerve fibers than the 2-day method on account of greater discreteness of the individual fibers and greater clarity of the background.

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THE PREPARATION OF PLASTER OF PARIS EMBEDDING BOXES

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There is a method of embedding with plaster of Paris boxes that does not seem to be commonly known, altho it has been used very successfully as a substitute for Guyer's[1] and Lee's[2] methods of embedding with paper boxes and Ferguson's[3] cellophane box. The technic is especially practical for large tissues and embryos, but serves equally well for small tissues. As the box is made of plaster of Paris, it is porous so that even within the box the paraffin is in contact with water, insuring even cooling, which results in a uniformly well-shaped

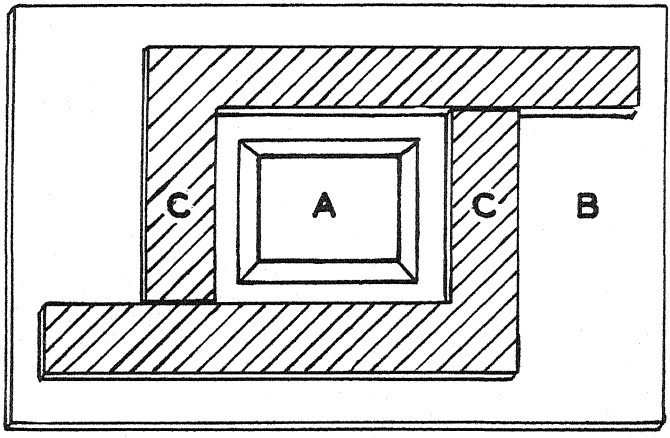

FIG. 1. Drawing of the mold used for preparing plaster of Paris embedding boxes. (A) Paraffin block, beveled, coated with a thin layer of vaseline, and sealed with the wide side down to (B), the copper plate. (C) Lead embedding angles. Any size box desired may be made by adjusting the embedding angles and size of the paraffin block.

block. Moreover, the method has the advantages of being very rapid and of saving the time one ordinarily uses in making paper boxes, for the box is permanent and needs only to be replaced when broken by rough handling. Boxes of various sizes can be made up at one's leisure (and will be ready to use whenever needed.)

The method of making them is as follows: Cut pieces of paraffin of

[1]Guyer, M. F. 1926. Animal Micrology, p. 37. Univ. of Chicago Press, Chicago, Ill.
[2]Lee, Bolles. 1928. Microtomist's Vade-mecum, pp. 74–75. P. Blakiston's Son & Co., Philadelphia, Pa.
[3]Ferguson, F. F. 1937. A cellophane embedding box. Stain Techn., 12, 71.

the size you desire the blocks. Bevel the paraffin block about 15° so that the bottom will be smaller than the top (Fig. 1, A) to allow the paraffin blocks to slip out when shrunk by cooling. Seal the block, wide side down, onto a copper plate (Fig. 1, B) or piece of cardboard and place around it two embedding angles (Fig. 1, C) about 1/8" from the widest part of the paraffin block. Coat the paraffin block with a thin layer of vaseline. Mix enough plaster of Paris and water to fill the mold and pour the mixture into the mold while still very wet. Allow the plaster of Paris to dry for an hour or more. Remove the embedding angles and when the plaster of Paris seems quite dry lift the box off the paraffin mold. When dry trim the box with a knife and sandpaper to the desired shape and thickness. It is advisable to leave the wall quite thick to prevent breakage.

To use the box: Soak it for a moment in the water to be used for cooling. Pick it out and pour out the water. Holding the box be-

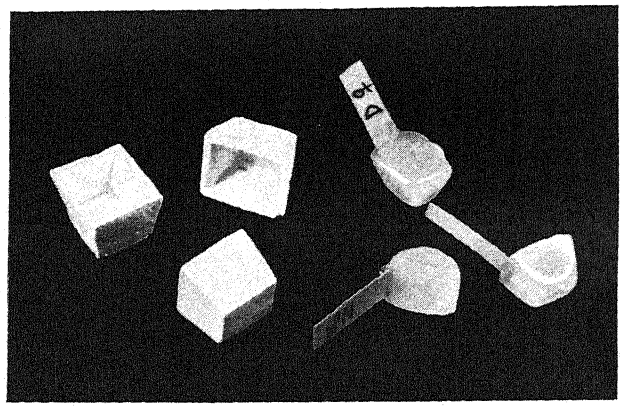

FIG. 2. Photograph of three plaster of Paris embedding boxes and three blocks made in them, showing how the blocks are labeled with a little paper tab.

tween the thumb and forefinger fill the box with melted paraffin using a medicine dropper. Drop the tissue into the melted paraffin. Orient tissue as desired with a warm needle or forceps. Insert a previously prepared slip of paper with identification marks on it into the melted paraffin at one end of the box (Fig. 2). Hold the box in the cold water to cool and blow gently on the surface until a thin film has congealed. Carefully lower the box under water and in a few seconds the paraffin will shrink from the sides of the box allowing the block to float free. The box may be used again immediately, but one can work faster by using two boxes alternately.

The use of plaster of Paris for making embedding boxes was first suggested to me by Margaret Long. Fred Schwind assisted in developing the technic for making them.

THE DEHYDRATION OF METHYLENE BLUE STAINED MATERIAL WITHOUT LOSS OF DYE

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ABSTRACT.—**A method is given for dehydrating methylene blue stained protozoan smears which should be applicable to the dehydration of tissues stained *intra vitam* with methylene blue. The procedure is: Wash with distilled water, place in tertiary butyl alcohol for 1 to 2 minutes, then in three or more changes of tertiary butyl alcohol for 15 minutes to an hour each, and mount directly in balsam or pass thru two changes of xylene before mounting.**

The problem of dehydrating methylene blue stained tissues without excessive loss of dye has engaged biologists for a long time. No entirely satisfactory technic has been found.

In the course of some studies, in the Department of Zoology at the University of California, on the large flagellate protozoon, *Trichonympha collaris*, it was necessary to dehydrate methylene blue preparations with a minimum loss of stain. The best method found in the literature was that of Cole.[1] After staining, Cole fixed the tissues in 10% ammonium molybdate, and passed them thru water, absolute-alcohol-*n*-butyl-alcohol, *n*-butyl alcohol, and methyl-salicylate-xylene to balsam. Altho fairly satisfactory, this method entailed the loss of a great deal of dye.

In a search for a more suitable method, it was observed that dehydration could be effected in tertiary butyl alcohol with the loss of an insignificant amount of methylene blue. Fixed and stained smears were washed in repeated changes of distilled water for 15 minutes to an hour. This washing does not remove the "combined" dye, but does take out that which is "uncombined." The smears were then placed in four changes of tertiary butyl alcohol. A small amount of dye comes out in the first change, hence it is inadvisable to leave slides in it for more than a minute or two. No dye comes out of the tissues in the next three changes. The smears may be left in them for from 15 minutes to an hour or longer, depending on their thickness. Allowance must be made for the fact that tertiary butyl alcohol penetrates about half as fast as ethyl alcohol. The slides were mounted directly in balsam, or passed thru two changes of xylene before mounting in balsam.

[1]Cole, E. C. 1936. A new methylene blue technic for permanent preparations. Stain Techn., 11, 45.

The above technic should be more successful in the dehydration of tissues stained *intra vitam* with methylene blue than the procedures heretofore used, since it extracts considerably less dye.

Smears of *Trichonympha* stained in aqueous solution with a number of other dyes were also dehydrated in tertiary butyl alcohol. The loss of dye was very slight or nil with toluidine blue 0, Nile blue sulfate, eosin Y, Ponceau 2R and orange G. Fair results were obtained with crystal violet.

The use of tertiary butyl alcohol as a general dehydrating agent was developed by Johansen[2] in a series of articles. It has all the advantages of dioxane, and is moreover both cheap and non-toxic. It is miscible with water, alcohol, balsam and paraffin. It should be borne in mind, however, that the freezing point of tertiary butyl alcohol is about 25° C., and this dehydrating agent must, therefore, be kept in a fairly warm place.

[2]Johansen, D. A. 1937–1938. Tertiary butyl alcohol methods. El Palo Alto News, **1**, 1–3, 5–6, 13–14, 21–22; **2**, 1–4.

A MODIFIED STAIN AND PROCEDURE FOR TREMATODES

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Trematodes are fairly easy to prepare as whole mounts, and there are a number of good staining methods by which they may be stained. The results usually desired are complete staining of the organs without stain in the parenchyma and muscle. If properly stained a sharp picture of the morphology and relationships of the organs results. It is unfortunate that stains and staining procedures are inconsistent, giving at one time and with one species of worm an excellent, sharp stain, and at another time or with another species a muddy, diffuse stain. The staining procedure described here has been found, over a period of two years, to give excellent and consistent results. It is sharper and less diffuse than any of the other carmine stains I have used, and is much more consistent than the hematoxylins. The stain is made up as follows:

To 100 cc. of 45% glacial acetic acid add 10 g. of carmine. Dissolve by heating and allow to come to a boil. Cool and filter. Carefully remove the filter paper from the funnel and spread it out flat to dry. The filtrate is Schneider's aceto-carmine and may be saved for the uses indicated for this stain. When the residue left on the filter paper is quite dry, remove it as carefully as possible. It may be used at once or placed in a tightly closed container for future use.

The staining solution is made up the same as Mayer's carmalum, substituting the acidified carmine described above for carminic acid.

Acidified carmine	1 g.
Alum	10 g.
Distilled water	200 cc.

The ingredients are mixed and dissolved by the aid of heat. When they are completely dissolved and cooled, filter the mixture and add a crystal of thymol to prevent mold growth.

If correctly used this stain gives a deep rose red color to the principal organs of trematodes. It is purely a nuclear stain, and consequently there is no diffuse stain in the cytoplasm, making the organs stand out in the practically transparent body. For best results the following procedure is suggested:

1. After the worms have been fixed and washed, if not already in water, they should be brought to water or not over 20% alcohol.
2. Place in the modified stain for 12–36 hours, depending on the size of the worm.
3. Remove from the stain and wash in one or two changes of water.
4. Dehydrate thru 20%, 35%, 50% to 70% alcohol.
5. The destaining is done in 70% alcohol, and is a very important step.

The best method I have found is the chlorine method of Mayer. This is done by placing a few crystals of potassium chlorate in the bottom of a small covered dish to which a few drops of concentrated hydrochloric acid are added. When chlorine is being given off from the mixture, fill the dish with 70% alcohol. This chlorinated alcohol is then used to destain the specimens. If deeply over-stained, the specimen may be immersed in the chlorinated alcohol, but for small specimens it is usually better to add the chlorinated alcohol to the alcohol covering them. In this way almost complete control is maintained over the destaining at all times. When adding the chlorinated alcohol to the specimen dish, care must be taken to disperse it evenly thruout the dish.

6. When the specimen is sufficiently destained (which can be determined only by trial and experience) remove the alcohol containing the chlorine and replace it with fresh 80% alcohol.

7. Finish the dehydration in the usual manner, and when completely dehydrated, add cedar wood oil to the absolute alcohol until the mixture is at least one half cedar oil.

8. Clear in cedar wood oil and mount in balsam.

LABORATORY HINTS

FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

J. A. de Tomasi — *Abstract Editor*

MICROSCOPE AND OTHER APPARATUS

RICHARDS, O. W. **A nomogram for the resolving power of microscope objectives.** *Trans. Amer. Micr. Soc.*, **57**, 316–18. 1938.

The nomogram consists of three scales: the wave length of light from 130–800 λ and the strong lines of the metals from cadmium to hydrogen, the numerical apertures from 0.07–2.0, and the resolving power of the system expressed in lines per inch and in microns. The scales are so placed that a straight line from the light employed thru the numerical aperture of the objective shows the resolving power. The value of monochromatic light in resolution becomes at once apparent. This is a convenient nomogram for anyone interested in critical microscopy.—*Virgene Warbritton Kavanagh.*

PHOTOMICROGRAPHY

BISHOP, F. W. **Exposure meter for cinephotomicrography and still photomicrography.** *Science*, **87**, 238–9. 1938.

Useful information is given on a scheme of setting up a photoelectric cell-galvanometer system. It is designed to gauge the light available in a microscope and to indicate the proper exposure. The cell is mounted in a fitting which slides over the microscope tube and holds it flat against it, or the ocular lens. All light is thus intercepted. Total cost of such unit is less than $30.—*J. A. deTomasi.*

MICROTECHNIC IN GENERAL

COLE, E. C. **Methyl methacrylate as a laboratory tool.** *Science*, **87**, 396–8. 1938.

This artificial plastic is insoluble in water, nearly insoluble in ethyl alcohol, amyl acetate, xylene, but readily soluble in chloroform and dioxan. A dioxan solution has been prepared which is colorless and can be used as a mounting medium. It dries more quickly than the common resins, and does not show discoloration on aging. No harmful action on stains has been observed and owing to its refractive index of 1.50–1.52, visibility is entirely satisfactory.

Rods of this material have found application as substitutes for expensive quartz rods in the illumination of living tissues. A rod ½ in. thick, tapered at the distal end and inserted in a lamp housing of a small 50 c.p. automobile bulb, furnishes enough illumination to permit examination of blood circulation in the vascular system of various organs.—*J. A. de Tomasi.*

CORDTS, H. M. **"Props" for cover glasses.** *Science*, **88**, 194. 1938.

In the preparation of embryo, or small insect slides, propping up the cover slip can be accomplished by using small bone curtain rings. They come in standard sizes fitting the usual round cover glasses. The ring is ground flat on both sides, and treated as a specimen ready for mounting. Wash in dist. water, run thru the alcohols, xylene, and soak and store in thin balsam. Before making a mount, let the ring adhere to the glass slip for 24–48 hr. After partial drying of the finished mount, paint the ring with heavy balsam.—*J. A. de Tomasi.*

MONK, C. R. **An aqueous medium for mounting small objects.** *Science*, 88, 174. 1938.

In this paper a medium is suggested which is capable of allowing mounting of minute parts of marine copepods so that an oil immersion lens can be used on them. It is a modification of Zirkle's Karo medium, as follows: white Karo syrup, 5 cc.; Certo, 5 cc.; water, 3 cc.; 1 crystal of thymol. When mounting, this medium begins to set in about 2 min.; regulate the velocity of setting by varying the water content. Dry by heat, and secure the cover glass with a drop of euparal.—*J. A. de Tomasi.*

DYES AND THEIR BIOLOGICAL USES

HAURY, V. G. **Modification of the titan yellow method for the determination of small amounts of magnesium in biological fluids.** *J. Lab. & Clin. Med.*, 23, 1079–84. 1938.

The author proposes a technic which eliminates objections to the older method based on Kolthoff's observation that Mg imparts a red color to alkaline solutions of titan yellow. In this proposed method, the determination of Mg depends upon a comparison between the excess titan yellow remaining in a standard and an unknown solution. He outlines methods for determining Mg in water, blood serum, and urine. The procedures are too detailed to be abstracted. In general, proteins are first removed by precipitation with trichloracetic acid; then Ca is removed with sodium oxalate. The dye is then added, mixed, centrifuged; the supernatant fluid is collected and the color compared with that of a similarly treated standard.—*P. R. Beamer.*

ANIMAL MICROTECHNIC

BANK, O. and KLEINZELLER, A. **Die Vitalfärbung des Fibroblastenzellkernes mit Kristallviolett.** *Arch. exp. Zellforsch.*, 21, 394–9. 1938.

The basic observation underlying this method of vital staining with crystal violet is the fact that the nucleus must be optically differentiated before it visibly begins to take up the dye. It is also possible that, from analogy of the theory of coacervation systems, optical differentiation or non-differentiation may be the result of a dehydration-hydration mechanism, whereby non-visibility correlates with maximum hydration of the nuclear colloids. The staining method is carried out in cover glass cultures of fibroblasts from the perichondrium of chicken embryo. It is found that crystal violet dissolved in the culture plasma is not adapted for vital staining even at 1:2,000,000 dilution. But the addition of electrolytes will permit staining, best results being obtained with Ca^{++}, and SO_4^{-}. This vital stain is reversible: it is obliterated by NH_4CNS, restored by $CaCl_2$. The nuclear stain is most successful when the nucleus is in telophase, the cells contain fat granules, and the cultures are two days old.—*J. A. de Tomasi.*

BRAGG, A. N. **The organization of the early embryo of Bufo cognatus as revealed especially by the mitotic index.** *Zts. Zellforsch. Mikr. Anat.*, 28, 154–78. 1938.

Yolky embryos section rather easily when embedded by the following modification of Hamlett's anilin method: Transfer embryos from 70% alcohol to aniline C.P., 1 part, 70% alcohol, 2 parts (2–6 hr.); anilin, 2 parts, 95% alcohol, 1 part, (2–6 hr.); anilin until clear, (approx. 1 hr.); 50% anilin, 50% toluene (1–6 hr.); 100% toluene, (1–3 hr.); sat. soln. 53° paraffin in toluene (1–4 hr.); melted 53° paraffin, 3–4 hr.; embed in 53° paraffin containing 1% bayberry wax if desired.—*Virgene Warbritton Kavanagh.*

BUCHER, O. **Untersuchungen über den Einfluss verschiedener Fixationsmittel auf das Verhalten des Schielddrüsenkolloids.** *Zts. Zellforsch.*, 28, 359–81. 1938.

The behaviour of the thyroid gland colloids of several mammalians, especially the rabbit, is investigated with particular reference to fixation in abs. alcohol, formalin, sublimate, picric acid, and OsO_4. It is found that fixation does not only affect the degree of colloidal shrinkage (and therefore capacity of staining) but reveals for each fixative definite shrinkage patterns. Fixation also influences

the appearance of vacuolization in the gland tissue: on this basis a differentiation is suggested between primary and secondary vacuoles. Staining of the marginal vacuoles has not succeeded. The isoelectric point of the thyroid colloids is also influenced by fixation: with formalin, pH 3.5; with alcohol, pH 4; with sublimate, pH 4.5; with picric acid, pH 5.5. A distinction between basophilic and acidophilic colloids cannot be established.—*J. A. de Tomasi.*

CHAUVIN, R. **La réaction de Feulgen-Verne chez le Criquet pélerin.** *Compt. Rend. Soc. Biol.*, **127**, 1075–6. 1938.

An account is given of the use of the Feulgen-Verne stain in demonstrating the organs of insects. The technic followed is not described. All nerve elements except the ganglia are colored deep violet. This may be evidence that the nerves of insects are myelinated. The Malpighian tubules are readily stained, certain of the tubes staining more deeply than others. In the alimentary canal, the musculature of the crop and gizzard and of the rectum between the rectal glands, the epithelia of the stomach, gastric caeca, and colon are stained. The illeum remains unstained. The deep staining reaction of the epithelia seems to uphold the common opinion that the digestion and absorption of fat begins in the stomach and gastric caeca. The pericardial cells are weakly stained, the stain being diffused in the protoplasm. The thoracic muscles stain strongly and the abdominal weakly. The fat body and hypodermis do not stain.—*F. J. Trembley.*

COCKE, E. C. **A method for fixing and staining earthworms.** *Science*, **87**, 443–4. 1938.

Histological slides of earthworms are often not quite as satisfactory as they should be. The following method of preparation is given: Clean the live specimen in water, feed (under moist cover) corn meal and agar 1:1; finely chopped lettuce may also be added. Change dish and food at intervals for 3 days. Cut specimen in ¾ in. portions and drop into 50° C. Allen's B-15. Fix 12 hr., rinse, and run thru 35, 50, 70, 80, 95, and 2 changes of 100% alcohol. Leave 1 hr. in each glass. Pass thru the following chloroform-alcohol combinations: 1:3, 1:1, 3:1, and chloroform, 1 hr. in each. Saturate chloroform with paraffin, leave 12 hr., add more paraffin, and put in oven at 58° C. for 6 hr. Pour off the liquid, add melted paraffin, and leave in oven 48 hr. Embed, and section at 12μ.

Stain 10–30 min. in Delafield's hematoxylin, wash, destain in 50% acid alcohol to a deep pink color, wash in NH_3-alcohol (5 drops NH_4OH to 100 cc. 50% alcohol) to a light blue color. Run up and mount as usual.—*J. A. de Tomasi.*

JOHNSTON, M. **Some methods of preparing teleost fish otoliths for examination.** *J. Royal Micro. Soc.*, **58**, 112–9. 1938.

Most otoliths require some degree of grinding on the convex surface before zones can be detected in them. For surface grinding and polishing, they are mounted, preferably with balsam, on the rounded end of a glass rod. The actual grinding is done with a razor hone made from Solenhofen lithographic slate; the polishing, with jeweler's rouge. Examination of otoliths, particularly thru the surfaces, is aided by the use of proper media of high refractive index. Best results are obtained with anilin (r.i. 1.580), aniseed oil (r.i. 1.557), and aq. solutions of chloral hydrate.

Sections of otoliths are prepared by a slow process of grinding, comparable to methods used with petrified paleontological specimens. A special grinder is here described suited for this type of material.—*J. A. de Tomasi.*

LISA, J. R. **A combined Gram-methyl-green-pyronin stain for formalin fixed tissue.** *Arch. Path.*, **26**, 728. 1938.

A method of staining as reported by Scudder and Lisa (Stain Techn., **6**, 1931) is brought up to date. The buffered crystal violet solution and National Aniline methyl green and pyronin dyes have been found stable and reliable for routine staining of paraffin sections. The work was performed with a batch of methyl green certified under the number NG-10 in 1932. Formulae and results are given in detail.—*Sara A. Scudder.*

LISON, L. **Etudes histophysiologiques sur les tubes de Malpighi des insectes. III. L'élimination des colorants basiques chez les Orthopteres.** *Zts. Zellforsch. Mikr. Anat.*, 28, 179–209. 1938.

This is one of a series of papers dealing with the reactions of living cells to various dyes. Dyes in concentrations from 0.05–0.5% were injected into the hemocoeles of three species by means of a fine glass pipet inserted between two abdominal segments. Basic dyes were more toxic than acid dyes, and smaller doses were used to avoid pathological changes. The animals were killed 2 min. to 2 hr. after injection. The tubes were examined immediately, in place, with a binocular and then studied immersed in mammalian Ringer soln. or hemocoele fluid on a covered slide.

Methyl green and malachite green were decolorized so rapidly in the animal that their distributions could not be followed. Thionin (Gr), brilliant cresyl blue (Gr), methylene blue (RAL), methylene green, toluidine blue (Gr), neutral red (microcolor), and Nile blue (Gr) stained only the vital granules in the tubes. Basic fuchsin (Gr), neuviolette 3R (C), crystal violet (Gr), and safranin O (Gr) stained the cytoplasm diffusely. Chrysoidine (Gr) and Janus green B (Meist) stained both granules and cytoplasm. The species varied in the staining gradients along the tubes, but showed similar gradients of reduction and metachromacy.—*Virgene Warbritton Kavanagh.*

VAN ROOYEN, C. E. **The micromanipulation and microdissection of the molluscum contagiosum inclusion body.** *J. Path. & Bact.*, 46, 425–36. 1938.

To make the tissue smear adhere to a cover slip, rub a few flakes of trilaurine onto the glass with an artist's paper pencil; pass the cover slip rapidly thru a Bunsen flame 3 times, wiping off excess grease with silk; then smear the fresh, moist tissue across the glass.—*S. H. Hutner.*

PLANT MICROTECHNIC

BHADURI, P. N. **Root-tip smear technique and the differential staining of the nucleolus.** *J. Royal Micro. Soc.*, 58, 120–4. 1938.

The paper discusses the examination of smears of cellular contents for cytological or other purposes. Heitz's Nukleal-Quetschmethode relies advantageously upon the use of the Feulgen reaction as a selective stain; but the chrom-osmic fixation preceding it is criticized. A new way of studying root tip smears is found in the use of a differential nucleolus staining technic. During development of this method various observations were made: for each plant, the optimum concentration of acetic acid and formalin as fixatives should be determined experimentally; with most plants, acetic alcohol (1:3) is quite a satisfactory fixative for the Feulgen stain, but interferes with the nucleolar stain; duration of hydrolysis is a critical point to be determined beforehand.

Smear Technic: Method I. Fix overnight in Navashin's, or acetic alcohol (1:3). Wash, and transfer to stoppered tubes containing *N* HCl. Transfer tubes for definite lengths of time to water at 58–60° C. Wash with dist. water, and hold in de Tomasi's decolorized fuchsin (Stain Techn., **11**, 137–44, 1936) 30 min. to overnight. Smear each root tip in a drop of 45% acetic acid between slide and cover glass. After 5 min. loosen cover glass by immersing horizontally in acetic alcohol 1:1. Transfer for 5 min. to acetic alcohol 1:9; soak 10 min. in two changes of 95% alcohol; treat 1 hr. in sat. soln. of Na_2CO_3 in 80% alcohol. Rinse, and keep 10 min. in 70% alcohol. Stain 15 min. with light green (Semmens and Bhaduri, Stain Techn., **14**, 1, 1939). Differentiate under the microscope in following solution: sat. Na_2CO_3 in 80% alcohol, 10 cc.; 80% alcohol, 40 cc. Rinse in 70%, dehydrate in 95, 100% alcohol. Clear in xylene alcohol, xylene, and mount in neutral balsam.

Method II. Hydrolize material stored in 80% alcohol with: 95% alcohol and conc. HCl, 1:1. Bring gradually to water, and stain as above.—*J. A. de Tomasi.*

CROSS, G. L. **An improved method of staining with fast green.** *Proceedings Oklahoma Acad. Sc.*, **17**, 69–70. 1937.

This procedure, developed for anatomical study of plant material is claimed to avoid plasmolysis, under- or overstaining, and loss of time. Kill and fix 24 hr. in following solution: formalin, 5%; glacial acetic acid, 5%; 70% alcohol, 90%. Wash 2 hr., with one change, in 80% alcohol. Run thru the following alcohol series: 1 hr. in 80% ethyl, 65%, and tertiary butyl, 35%; 1 hr. in 90% ethyl, 45%, and butyl, 55%; 1 hr. in abs. ethyl, 25%, and butyl, 75%; 2–3 hr. in butyl, changing each hour. Infiltrate overnight on top of oven. Replace butyl alcohol with 6 changes of paraffin in 6 hr. Pass slides thru: xylene, 6 min.; abs. alcohol, 2 min.; 95% alcohol, rinse 5–6 times; 50% alcohol, rinse 5–6 times. Stain 6–12 hr. in 1% safranin in 50% alcohol. Wash in water and run thru: 50% alcohol, 2–3 min.; 70, 95, 100% alcohol, 40% abs. alcohol and 60% xylene, 5–6 rinses in each. Counterstain 2–4 min. with a few cubic centimeters of sat. clove oil soln. of fast green in 40% abs. alcohol and 60% xylene. Rinse 5–6 times in 40% abs. alcohol and 60% xylene, clear 5 min. in xylene, and mount.—*J. A. de Tomasi.*

GRIGG, F. C. **On the use of dioxan as a dehydrating medium.** *J. Queckett Micro. Club.* Series 4, **1**, 4–11. 1938.

For cytological material from plants, the following dioxan schedule has proved of advantage: 10%, 1 hr.; 20%, 2 hr.; 30%, 4 hr.; 40%, overnight; 50%, 4–5 hr.; 60%, 4–5 hr.; 70%, 24 hr.; 80%, 24 hr.; 90%, 24–36 hr.; 100%, 48 hr. After the last bath, introduce $CaCl_2$ for dehydration. Remove to another vessel with fresh dioxan at 25° C. and add a few drops of xylene every 15 min. occasionally removing part of the mixture. In 6–7 hr. material will be in pure xylene. Infiltrate with low melting point paraffin: beeswax, 1 part; paraffin, 6–10 parts at from 46° to 48° C. Cutting must be done at about 52° F.—*J. A. de Tomasi.*

PALMQUIST, E. M. **The simultaneous movements of carbohydrates and fluorescein in opposite directions in the phloem.** *Amer. J. Bot.*, **25**, 97–105. 1938.

Uranin is used in order to determine whether two different solutes can move simultaneously in opposite directions in the same phloem tissue. This water soluble Na salt of fluorescein is chosen instead of other fluorescein compounds because it seems to exercise no harmful effect on phloem tissue. The dye is introduced by immersing the terminal leaflet half way in a 0.1% aq. soln. Control of the progress of the dye is accomplished by cutting cross sections of the petiole of the treated leaf, and examining microscopically for fluorescense in a beam of ultraviolet light. It is proved that carbohydrates (sugars) move out of leaves thru the phloem, while fluorescein is entering.—*J. A. de Tomasi.*

MICROÖRGANISMS

ASHBY, G. K. **Simplified Schaeffer spore stain.** *Science*, **87**, 443. 1938.

This is a technic intended to facilitate the staining of bacterial spores by beginners. Dry the smear over a Bunsen burner. Heat on steam bath until water condenses in droplets on bottom of slide. Flood with 5% aq. malachite green, and heat 1 min. Drop and rinse in cool water. Counterstain 30 sec. with 0.5% aq. safranin, and rinse again. The spores stain green; the vegetative forms, red.—*J. A. de Tomasi.*

BAILEY, H. D. **A practical stain for the spirochetes of syphilis and Vincent's angina.** *J. Lab. & Clin. Med.*, **23**, 960. 1938.

In cases where dark-field tests failed to reveal them, smears stained by this method show the organisms plainly. *Procedure:* A direct smear of syphilis serum or tissue juice is made on a clean slide. In case of Vincent's angina, the ulcerated material is spread in a loopful of water. Air dry and fix with heat. Cover with $N/20$ HCl, 10 sec.; wash in running water, 5 sec.; cover with Gram's iodine solution, 5–10 sec.; wash; apply anilin-gentian violet, 5–10 sec.; wash; apply Gram's iodine solution, 5–10 sec.; wash; apply anilin-gentian violet, 5–10 sec. Wash, blot and examine. Gentian violet without anilin oil may be used. By applying the iodine and gentian violet a third time a very deep color is secured. The stain is not permanent.—*P. R. Beamer.*

GOLDBERG, L. H. **A rapid method for staining blood smears. A modification of the original Wright technique.** *J. Lab. & Clin. Med.*, **23**, 959. 1938.

This modification of Wright's technic is employed where time is a factor. A thin to medium smear is made and placed across the edges of an ordinary drinking glass filled ¾ full of dist. water. Cover the smear with Wright's stain for about 10 sec. Ignite the stain and allow to flame 10–20 sec. By use of a small rod the flaming slide is then dropped into the dist. water. Remove, dry and examine. Nucleated cells stain deeply and in much more detail than in the original procedure. Red blood cells are stained poorly.—*P. R. Beamer.*

HENDEY, N. I. **An efficient technique for cleaning diatoms.** *J. Royal Micro. Soc.*, **58**, 49–52. 1938.

The classification of diatoms is based on the interpretation of the silica frustules that make up the skeleton of the organisms. Cleaning must remove both the coleoderm and the protoplasmic contents of the cells. The chemical cleaning is carried out in a closed apparatus intended to take care of both cleaning and bleaching, with the added advantage of neutralizing the obnoxious fumes evolved. The operation is based on the heating of diatom material with HNO_2 and H_2SO_4 in a closed flask, a very short time for fresh material, boiling 3–10 min. for fossil material. Upon cooling, a $KClO_3$ soln. is introduced with great caution. Products of interactions include Cl_2 which is subsequently neutralized by allowing it to mix with a Na_2SO_3-Na_2CO_3 soln.—*J. A. de Tomasi.*

MIHAELOFF, S. **Comparative research in the different methods of staining the gonococcus.** *Revue Medicale Francaise*, **18**, 609–717. 1937.

A brief description of the appearance, morphology and cultural requirement of the gonococcus are given, along with a detailed review of the Gram technic from time of Gram and Roux. Formulae for various counterstains, single stains such as Loeffler's, and double stains such as Unna-Pappenheim are given. Double staining is defined as one which colors the cells in contrast to the bacteria. Emphasis is laid upon stained films as a necessary aid to diagnosis of gonorrhoea; for this purpose the smears should be prepared either by dilution of thick pus with water or by smearing thinly streaked films. It was concluded that the Gram staining technic is of chief importance for differential diagnosis and control of other methods for staining exudates. Recognition of gonococci in films was facilitated by procedures such as that of Galli-Valerio, but procedures such as the original Pappenheim and Pick-Jakobsohn gave inconstant results. No specific stain for the gonococcus was found.—*Sara A. Scudder.*

PIEKARSKI, G. **Studies on the cytology of paratyphus and coli bacteria.** *Arch. Mikrob.*, **8**, 428–39. 1937.

For demonstrating the "nucleoid" contents of the cell of these bacteria the fresh smear on a slide was fixed and stained in aceto-carmine, or was fixed in acetic acid, osmic acid, Carnoy's or sublimate alcohol and subjected to the Feulgen reaction as follows: After fixation, rinse in dist. water. Hydrolize about 10 min. in *N* HCl at 60° C. Rinse in dist. water. Stain for about 1 hr. in fuchsin sulfurous acid with an excess of SO_2. Wash in SO_2 in water (about 10–15 min., changed twice). Run up thru alcohol, xylene, and Canada balsam.—*Merritt N. Pope.*

SHIH, L. **Über den Heterothallismus des Staubbrandes, Spacelotheca cruenta (Kühn) Potter, der Mohrenhirse Andropogon sorghum Brot.** *Arch. Mikrob.*, **9**, 167–92. 1938.

In order to study the cytology of the germination stages, chlamydospores of *S. cruenta* are germinated in a hanging drop of dist. water, 2% glucose or other nutrient soln., and treated as follows: Nearly dry the drop in the air and fix for at least 24 hr. in a single drop of the strong chromo-aceto-osmic-acid of Flemming, wash thoroly, dry, and place for 12 hr. in ether to extract all fats. Cover the culture with a soln. of 2% celloidin in a mixture of ether, 70%; abs. alcohol, 30%. Let liquid evaporate, mordant in 4% iron alum and stain in 0.5% hematoxylin.—*Merritt N. Pope.*

TABET, F. **A modification of Wilson and Blair's bismuth medium suitable for both typhoid and paratyphoid bacilli.** *J. Path. & Bact.*, **46**, 181–93. 1938.

This experimental study of the Wilson-Blair medium for the detection of typhoid and paratyphoid B as black colonies in the presence of many *B. coli* demonstrated that (1) the bismuth is mainly responsible for the blackening (due to Bi_2S_3), and also helps inhibit *B. coli;* (2) the selectivity is mostly due to the brilliant green.

The modified medium is prepared with 3 sterile flasks A, B and C: In A place 6 g. bismuth ammonio-citrate (Scales); in B, 10 g. anhyd. Na_2SO_3; in C, 10 g. glucose. To flask A add 50 cc. boiling dist. water and shake till the bismuth ammonio-citrate is dissolved; add 1 cc. 5*N* NaOH, mix well and cool. To B add 100 cc. boiling dist. water and cool. To C add 50 cc. cold dist. water; boil 1 min. and cool. Mix contents of A and B, add 3.5 g. anhyd. Na_2HPO_4, boil about 30 sec., cool, add contents to C. This stock mixture keeps for several months in the dark at room temperature. Shake thoroly before use.

The medium: To 100 cc. of melted nutrient agar at about 80° C. add 20 cc. of the stock mixture; 1 cc. of 8% $FeSO_4 \cdot 7H_2O$ in dist. water and 1 cc. of 0.5% brilliant green in dist. water, with vigorous shaking after each addition. Pour into plates and use as fresh as possible. The medium may be stored about 5 days in the refrigerator but deteriorates rapidly at room temperature. The pH is usually between 7.9–8.0; adjustment is unnecessary.—*S. H. Hutner.*

WINOGRADSKY, S. **Etudes sur la microbiologie du sol et des eaux (neuvième mémoire). Sur la morphologie et l'oecologie des Azotobacter.** *Ann. Inst. Pasteur*, **60**, 351–400. 1938.

A technic is offered to avoid overstaining the background when staining colonies of Azotobacter in soil on silica gel plates. Stain in 1% fast acid blue (violamine) (recommended by Romell, Stain Techn., **9**, 141. 1934), phenol 0.5% for about 30 sec. This acts more as a mordant than a stain. Wash off first stain then apply a weak solution of "gentian" for 30 sec.

If the culture is old, containing cysts, heat the drop of acid blue until it just begins to steam, then stain 1 min. in "vesuvine", rinse, stain quickly with "gentian". In successful preparations the central body of the cyst is brown or black, the outer layer of the capsule is blackish, the inner yellow.

Mounting in balsam causes bad shrinkage. Instead, dry the slides, wrap in silk, and examine in water as required. Preparations keep their freshness.—*S. H. Hutner.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the Oct. number of this journal.

STAINS CERTIFIED SEPT. 1, 1938 TO NOV. 30, 1938*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Crystal violet	NC—19	90%	As histological, cytological, and bacteriological stain; and in bacteriological media	Sept. 26, 1938
Basic fuchsin	NF—31	88%	For general staining, the Feulgen reaction, and in bacteriological media	Sept. 30, 1938
Methylene blue	MA—1	85%	As histological and bacteriological stain; and as constituent of blood stains	Oct. 4, 1938
Nile blue A	LNb—1	73%	As stain for fats and fatty acids	Oct. 10, 1938
Phloxine	NPh—8	84%	For histological counterstain	Oct. 17, 1938
Methyl green	CG—8	60%	In histology and as constituent of Pappenheim stain	Oct. 19, 1938
Sudan IV	LZ—1	68%	As fat stain	Oct. 19, 1938
Crystal violet	BC—1	89%	As histological, cytological and bacteriological stain; and in bacteriological media	Oct. 20, 1938
Azure A	NAz—6	90%	As histological stain and as constituent of blood stains	Nov. 7, 1938
Fast green FCF	NGf—4	90%	As histological counterstain	Nov. 12, 1938
Acid fuchsin	NR—11	62%	As histological stain, and for use in Andrade indicator	Nov. 17, 1938
Indigo carmine	CI—5	90%	As histological stain	Nov. 30, 1938

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each due for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

STAIN TECHNOLOGY

VOLUME 14 APRIL, 1939 NUMBER 2

PROGRESS IN THE STANDARDIZATION OF STAINS

STAINING SOLUTIONS INCLUDED IN THE NATIONAL FORMULARY

Altho most users of stains do not realize it, recent editions of the National Formulary, published by the American Pharmaceutical Association, have contained formulae for staining solutions in a source that is available to druggists fairly generally thruout the United States. Unfortunately, however, no effort has been made in the past to keep these formulae up to date or to even make certain that they were entirely correct. This, of course, has been due to the fact that few members of the Pharmaceutical Association understand staining and very few biologists consult the National Formulary.

This situation is to be changed in the future. A representative of the American Pharmaceutical Association, Dr. Louis Gershenfeld, has been appointed on the Stain Commission and the chairman of the Commission has been made a member of the National Formulary committee. This arrangement results in full cooperation and two or three conferences have been held with the purpose of standardizing the staining formulae in the next edition of the N. F. and taking care to see that they agree with those recommended by the Stain Commission. It will be some time yet before this new edition of the Formulary is out[1] and still longer before it becomes official; eventually, however, this should result in reliable staining formulae being at the disposal of druggists anywhere in the country. This ought to make it possible for a physician requiring a small quantity of some staining solution to obtain what is needed from his druggist in comparatively reliable quality.

Another result which is to be expected from this cooperation between the Stain Commission and the National Formulary committee is a more official recognition of the former than it has ever had. The new edition of the Formulary will specify stains that have been certified by the Commission on Standardization of Biological Stains.

[1]The chapter on Materials and Preparations for Diagnostic Use is now out in preprint form, and may be obtained from the Amer. Pharamaceutical Assoc., Washington, D. C. Price $1.50.

Inasmuch as Federal and state legislation very generally specify National Formulary quality in connection with drugs and related products, this gives a semi-official standing to an organization whose specifications and approval are recognized in the Formulary.

It is felt, therefore, that this cooperation is likely to have advantages for users of stains in small quantities and that it will also add to the prestige of the Stain Commission.

—H. J. Conn.

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the January number of this Journal.

Stains Certified Dec. 1, 1938 to Feb. 28, 1939*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Crystal violet	LC–14	94%	As histological, cytological, and bacteriological stain; also in bacteriological media	Dec. 7, 1938
Wright stain	NWr–12	....	As blood stain	Dec. 11, 1938
Alizarine Red S	NAr–2	....	As stain for bone	Dec. 14, 1938
Safranin O	NS–11	89%	As histological, cytological and bacteriological stain	Dec. 27, 1938
Carmine	CCa–5	....	As histological and cytological stain	Dec. 27, 1938
Hematoxylin	FH–14	....	As histological and cytological stain	Jan. 20, 1939
Eosin B	LEb–1	87%	As histological counterstain	Jan. 23, 1939
Methylene blue	CA–17	86%	As histological and bacteriological stain; and as constituent of blood stains	Feb. 3, 1939
Basic fuchsin	CF–17	92%	For general staining, Feulgen stain, and in bacteriological media	Feb. 8, 1939

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each due for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

DISSECTION AND PREPARATION OF WHOLE MOUNTS OF ENDOSPERM FROM THE SEEDS OF *GREVILLEA* (PROTEACEAE)

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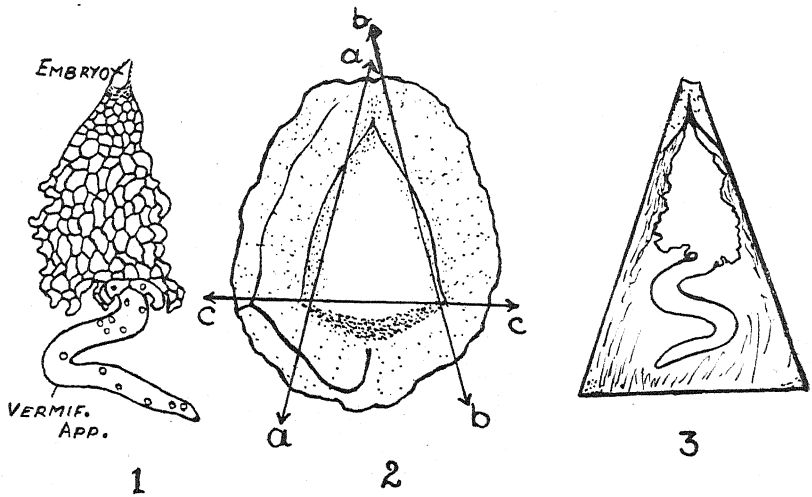
ABSTRACT.—It is sometimes very desirable to supplement the examination of serially cut microtome sections by dissection and study of whole mounts of objects. In the case of the seeds of *Grevillea* the endosperm forms a curious worm-like structure, the *vermiform appendage*, in elucidating the nature of which microtome sections alone are inadequate. The dissection of the endosperm for demonstrating this structure is very necessary; otherwise the structure is altogether overlooked. In dissecting out the endosperm, which is easily done, either fresh seeds or previously preserved seeds may be used; the former are easier for handling and yield more satisfactory results. In the case of fresh material, an immediate killing is necessary and any one of the ordinary killing fluids may be used; Bouin's fluid was tried by the writer and was entirely satisfactory. After killing, the material is stained and dehydrated, the several stages of which are best done on the slide alone on which the material is placed. Mounting is done in any of the mounting media, and Canada balsam answers the purpose quite well.

While it cannot be disputed that with the introduction of the microtome, great facilities are available to the modern plant morphologist for cutting uniformly thin serial sections for examination of minute details, the value of dissection for preparing whole mounts for study may sometimes become surprisingly significant. Recently, Buchholz[1] has demonstrated that in the case of conifer embryos serial microtome sections alone fail to reveal a complete and intelligible picture of the tortuous suspensor systems and has stressed the importance of dissecting out the embryos and preparing whole mounts by which the coiled suspensor can be easily followed and studied. The present note is intended to point out another instance where an examination of microtome sections needs to be supplemented by a study of whole mounts prepared by dissecting the endosperm from the seed in *Grevillea*, a plant belonging to the Proteaceae.

[1]Buchholz, J. T. 1938. Dissection, staining, and mounting of the embryos of conifers. Stain Techn. **13**, 53–64.

The endosperm in *Grevillea* shows an upper cellular and a lower cenocytic portion. The latter forms a highly coiled tubular structure, worm-like in appearance and called the *vermiform appendage* of the endosperm, which was for the first time discovered by the writer and is elsewhere fully described.[2] Serial microtome sections of the seed, even when cut fairly thick, are not entirely satisfactory nor adequate for determining the exact nature of this coiled structure. Such preparations merely show bits of the *appendage* distributed in a number of sections. A dissection of the endosperm, which can be mounted whole, is on the contrary very desirable to get a full understanding of not only the relations between the cellular portion and the non-cellular coiled structure, but also in determining exactly the true nature of the coiled structure itself. (Fig. 1).



Figs. 1 to 3. Dissection of Endosperm from the seeds of *Grevillea*. *Fig. 1:* A whole mount of endosperm dissected out of a seed of *Grevillea robusta* showing the *vermiform appendage*. ×14. *Fig. 2:* An entire seed with the broad wing; the lines indicate how the margins may be cut away by a scalpel. ×3. *Fig. 3:* One piece of the seed coat (seen from inside) after cutting away the margins of the seed as indicated above; the whole mass of endosperm with its *vermiform appendage* is seen loosely hanging from the micropyle and may be lifted up on the point of a mounted needle. ×12.

In preparing whole mounts of the endosperm, it is important to procure seeds at the right stage. The pericarp of the fruit containing such seeds is quite green in color and the embryo inside the seeds is small and consists of a spherical or oval mass of cells in which the differentiation into the histogenic layers has just become recognizable.

[2]Kausik, S. B. 1938. Studies in the Proteaceae. I. Cytology and floral morphology of *Grevillea robusta* Cunn. Ann. Bot. N.S., **2**, 899-910.

The seeds in *Grevillea* are quite flat and are, therefore, easily and securely placed on the slide for dissection. The margins of the seed are cut away as shown in Fig. 2 and the seed coat is then lifted off in two pieces. This process is easily accomplished as the tissue inside the seed coat is almost completely disorganized and a large cavity is formed. The mass of endosperm is freely suspended inside this cavity. After the seed coat is removed, this mass of endosperm usually lies loosely adhering to one of the two pieces (Fig. 3) and may be easily picked up on the point of a fine mounted needle and placed on a clean slide for further treatment and mounting.

The dissection of the endosperm may be performed either with fresh or with preserved seeds. The former are always preferable for dissection, whereas the preserved material shows the endosperm a little crushed and shrunken. After the mass of endosperm is completely removed and placed on the slide, the following procedure in killing, staining and mounting may be followed. At all stages in this procedure it is always best to retain the material on the slide alone, on or from which drops of various fluids may be added or withdrawn as necessary by means of an ordinary medicine dropper.

Killing and Staining

If the material is fresh and not previously preserved the mass of dissected out endosperm needs to be killed in any of the ordinary killing fluids. Bouin's fluid was used by the writer and the results obtained were entirely satisfactory. The killing is over in about an hour after which the material is washed in three or four changes of distilled water and then stained. If Heidenhain's iron-alum hematoxylin is used, as was done by the writer in most cases, a preliminary mordanting in 4% iron-alum is necessary, after which the material is again washed in changes of water and stained for about two hours. The material is then destained in 2% iron-alum, washed in water and dehydrated in grades of alcohol and finally brought up into pure xylene, the entire process being over in about an hour. The material is next ready for mounting which is quite satisfactorily done in clear and fairly thin Canada balsam.

In the case of material which was previously in some preservative, as for example in formalin-acetic-acid-alcohol, the material is first washed thoroly in several changes of 50% alcohol, brought down to water gradually and is then subjected to the same treatment as in the case of fresh material outlined above. As previously stated, however, it is preferable to have fresh material, as the results are more satisfactory.

Instead of hematoxylin, safranin or any other stain may also be used, either singly or in the usual combinations. In the case of safranin stained material it is always good to retain a little more stain than is actually required at the end, as the excess of stain is lost in passing the material thru the grades of alcohol while dehydrating. If Venetian turpentine is employed in place of Canada balsam, in mounting the material and sealing, unnecessary labor and careful watching are involved; Canada balsam answers the purpose well.

The dissection of endosperm for whole mounts was first successfully tried by the writer in *Grevillea robusta* and was subsequently followed in the case of a few other species of the Proteaceae, namely *Grevillea banksii, G. hilliana* and *Macadamia ternifolia.* In the first two the presence of the *vermiform appendage* of the endosperm was again noticed, while in *Macadamia*[3] its place and function were found to have been taken over by the formation of irregular multinucleate lobes of the lower region of the endosperm. Yet another species, *Hakea saligna,* was also examined, but the seeds that were available were rather old and the endosperm was completely used by the embryo in these. This will be taken up again at the earliest opportunity.

The earlier workers in the family, including Messeri,[4] who investigated *Grevillea macrostachya* and Brough,[5] who worked on *Grevillea robusta* have failed to demonstrate the existence of the *vermiform appendage* of the endosperm; and these, in all probability, confined themselves to an examination of microtome serial sections alone and thus overlooked an important feature of the endosperm. It is undoubtedly of great interest to find out if similar or corresponding features are present in the other members of the Proteaceae. It is the intention of the writer in contributing this brief article that interested readers, who may be able to secure seeds of any of the Protead genera, will look for this peculiar feature in the development of the endosperm.

[3]Kausik, S. B. 1938. Studies in the Proteaceae. II. Floral anatomy and morphology of *Macadamia ternifolia* F. Muell. Proc. Ind. Acad. Sc., **8**, 45–62.

[4]Messeri, A. 1928. Embriologia di *Grevillea macrostachya* Brongn. et Gris. Nuovo Giorn. Bot. Ital., Nuova serie, **34**, 1037–42.

[5]Brough, P. 1933. The life history of *Grevillea robusta* Cunn. Proc. Linn. Soc. N.S.W., **58**, 33–73.

COMPARISON OF METHODS FOR DEMONSTRATING GLYCOGEN MICROSCOPICALLY

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ABSTRACT.—Three methods for the microscopic demonstration of glycogen are compared: namely, Best's carmine, the iodine method, and Bauer's modification of the Feulgen technic. The lability of glycogen necessitates immediate fixation, small pieces of tissue, and agitation of the fixative. The fixative recommended consists of 9 parts by volume of absolute ethyl alcohol to one part of 40% formaldehyde neutralized with $MgCO_3$; the alcohol in this formula may be saturated with picric acid if desired. After fixing, the tissue should be washed in absolute alcohol and embedded in paraffin or celloidin, taking care to avoid overheating of blocks or sections. A sharp knife should be used in sectioning. The Best's carmine technic is employed by staining first with hematoxylin, then for 20 minutes in: Best's stock solution (prepared by the usual formula, using Grübler's carminum rubrum optimum), 10 cc.; concentrated ammonia, 15 cc.; methyl alcohol, 30 cc.; used fresh after agitating and without filtering. The iodine method calls for Lugol's aqueous iodine. The Bauer-Feulgen method calls for sections mordanted 1 hour in 4% aqueous CrO_3 and washed in water 5 minutes, followed by 10-15 minutes in: a suitable basic fuchsin (e. g. Grübler), 1 g.; warm dist. water, 100 cc., with filtration of solution while still warm; normal HCl, 20 cc., added after cooling; $NaHSO_3$, 1 g.; ripened to a straw or amber color for 24 hours in the dark. After this bath the sections are rinsed 1½ minutes each in 3 changes of M/20 $NaHSO_3$, then 10 minutes in running water, and are then counterstained with hematoxylin. Of these three methods the iodine technic proves a good check on either of the others. Saliva digested controls are also essential, taking care to wash out mucus from sections.

From time to time one sees in the literature statements as to the relative accuracy and dependability of the various methods for the detection of glycogen in microscopic sections. For many years we have been studying glycogen histologically and some results have been published. Numerous requests have been received for information relative to the fixing and staining of glycogen in tissues; and this seems to be an opportune time to place our experience at the disposal of others engaged in similar studies.

Before proceeding to a discussion of the staining methods, it is important to emphasize the fact that glycogen in fresh tissues is a

labile material. It may disappear rapidly from many tissues post mortum in part or completely. For this reason prompt fixation is essential; the tissue should be in the fixative in less than 10 minutes after the cessation of the blood flow. It is important to use small pieces of tissue (2–3 mm. thick) thru which the fixing fluid can rapidly penetrate.

While in the fixing fluid the tissue should be moved about frequently but gently. A piece of tissue lying in the bottom of a dish of strong alcohol remains in a dilute alcohol solution for hours unless agitated. The agitation also allows the fixing fluid to penetrate from all sides at once and so completes the penetration of the block in less time than otherwise.

We have found three fixatives reliable, that is, they always preserve a maximal amount of glycogen when used as we have indicated.

A—Absolute ethyl alcohol
B—Absolute alcohol ..... 9 parts (by volume)
Pure 40% formaldehyde solution neutralized with $MgCO_3$ ... 1 part
C—Absolute alcohol saturated with picric acid ..... 9 parts
Neutral formaldehyde solution ..... 1 part (Lison 1936)

The sections should be cut with a sharp knife and mounted very flat and smooth. This is particularly important for Best's carmine (see p. 49).

Four staining methods are of special value.

Best's Carmine

The stock solution is prepared as follows:

Grübler's carminum rubrum optimum	2 g.
Potassium carbonate	1 g.
Potassium chloride	5 g.
Distilled H_2O	60 cc.

Boil gently until the color darkens. Allow to cool. Add 20 cc. concentrated ammonia. Ripen 24 hours.

In making up this formula it is important to select a carmine that will stain glycogen; as some will not. It is possible, of course, by chemical treatment to modify a carmine that is unsuitable for this formula so as to make it work, but since most biologists are not familiar enough with the chemical processes involved, it saves time to select a carmine that is satisfactory and use it exclusively. After trying a large number, some satisfactory, some not, Grübler's carminum rubrum optimum has proved uniformly satisfactory. For use the solution is prepared as follows:

To 10 cc. of the stock solution add 15 cc. of concentrated ammonia and 30 cc. of pure methyl alcohol. Stir thoroly and use at once. *Do not filter.*

This will stain the glycogen a brilliant vermilion-red color, sometimes with a metallic gleam. It is used as follows:

The sections, cut in paraffin and fastened to slides with albumin, are brought down to water, then stained with a hematoxylin solution suitable for a nuclear stain. We use R. R. Bensley's modification of Ehrlich's hematoxylin.

They are then put in the freshly diluted Best's carmine solution for 20 minutes. If glycogen is present this is enough time and does not give too much chance for the background to become pink, if that tendency is present. When testing a new tissue for glycogen it is well to run a section of properly fixed liver or the like along with the first lot of sections stained in a given batch of stain to make sure that the stain is working properly.

The sections are then rinsed in about three changes of pure methyl alcohol. In the first washing a good bit of precipitate comes off the slides, so this alcohol should not be used again. They may now be cleared and mounted in the ordinary way with absolute alcohol and xylene, but we prefer to use acetone for dehydration and toluol for clearing.

In spite of precautions indicated above, one of the difficulties to contend with in the use of Best's carmine is the frequent adherence of precipitate to the sections. One soon, however, becomes fairly proficient in distinguishing this from glycogen. Another trouble is a tendency of the sections to develop a pink tinge all over, even in the nuclei which tho previously well stained in hematoxylin may change color in the carmine. Lacking a sharp color contrast between the various cell constituents, material of this type is most difficult to study even tho vermilion-red glycogen is present, and in these the salivary digestion controls help very little. So far we have not been able to do much about this difficulty. Apparently it is due to something inherent in the material or impurities in the fixing or dehydrating reagents and not to faulty staining solutions. It has occurred with various types of tissue supposedly promptly fixed, and a specimen that gives this result will do so even when sections from the same block are obtained years apart. Here, too, prompt fixation of the tissue may make a difference.

If glycogen in a specimen fails to stain with Best's carmine, pay more attention to the freshness of the solutions. One cannot tell how long such a colloidal solution will keep but the stock should remain satisfactory for some weeks and the dilute solution at least a few days. It seems preferable to make a fresh solution of the latter each time it is needed. Also, solutions from older stock will some-

times stain only part of the glycogen present and this may be a paler shade. Such solutions gradually lose their glycogen staining capacity entirely.

The Iodine Method

We use this method for the most part as recommended by Gage, but with some modifications for ease and accuracy of use, with our particular material.

Iodine colors glycogen a reddish-brown if it can reach it, but during the section mounting process factors may appear which keep the tissue from taking up the iodine, even when there is plenty of glycogen present.

Mount the sections in the usual way, being very careful not to heat them more than absolutely necessary to remove wrinkles, dry, remove paraffin with xylene or benzol, transfer thru alcohols to water, leave 10 or 15 minutes in a Lugol's aqueous iodine solution, and allow to dry in the air after blotting with filter paper. They must be protected from dust and not left too long before mounting or they will fade again. When they are dry, mount in yellow vaseline, as recommended by Gage, being very careful not to heat them too much.

Drying the sections does perhaps distort the cells somewhat, and air bubbles from the dried section work out into the mounting medium, but after a day or two they are relatively clear, and the glycogen results are uniformly good. Any preparations made in this way are not generally so good for high-power study as those made with Best's carmine or with the Bauer-Feulgen method, but nevertheless it is a useful control for other methods.

Gage recommended two methods of using iodine for glycogen. One may use either aqueous or alcoholic Lugol instead of water for spreading the paraffin sections. If this is done it is as important not to overheat the sections as it is when using the method recommended above, else they may not give the reaction with either aqueous or alcoholic iodine. For more permanent preparations, place the dried paraffin sections directly into alcoholic Lugol without removing the paraffin, dry, remove paraffin with xylene, and mount in yellow vaseline. This method, in my experience, is apt to give very variable color reactions in different sections of a series mounted on a single slide even when care has been taken not to overheat any part of the slide.

The Bauer-Feulgen Method

This test consists in the application to glycogen of the Riche and Bardy reaction for aldehydes as modified by Schiff and later by Feulgen. It consists in freeing aldehydic groups by a moderate

hydrolysis and then detecting them with a solution of basic fuchsin decolorized by sulfurous acid. A good discussion of it as applied to glycogen may be found in Lison (1936, p. 231). It may be called the Bauer-Feulgen test for glycogen as it is actually the Feulgen method which Bauer modified for specifically staining glycogen.

The Feulgen reagent is made as follows:

Dissolve 1 g. of basic fuchsin[1] in 100 cc. dist. water by heat. Filter warm and add to the filtrate, when cool, 20 cc. of normal HCl. Add 1 g. of $NaHSO_3$. Allow the solution to stand 24 hr. before using, when it should be a pale straw color.

Sections cut in paraffin are mounted on slides in the usual way, put in 4% chromic acid for 1 hour, or 1% chromic acid overnight. Wash in running water for 5 minutes. Place in the Feulgen reagent for 10–15 minutes. Then rinse in 3 changes of a molecular solution of $NaHSO_3$ one part, tap water 19 parts. The sections should remain 1½ minutes in each change. Wash in running water for 10 minutes.

One may now clear and mount the sections, or the latter may be first counterstained with a good nuclear stain. For this purpose Ehrlich's hematoxylin (Bensley, 1938) has been used with good success in this laboratory.

If the technic is successful, glycogen should be stained a deep red color with a violet tinge, and if the hematoxylin has been used for counterstain the nuclei will be a clear lavender color.

In both the Best's carmine and Bauer-Feulgen methods, particularly in the latter, it is advisable to agitate the slides frequently in all solutions to insure even distribution of color in different sections on the slide. An extended study shows that a failure to do so may result in considerable color difference between adjacent serial sections.

Saliva Digestion

Since none of the three methods described stains glycogen exclusively, no study of the distribution of glycogen in tissues is complete without the saliva digestion control.

Each time a new lot of material is stained with either the Best's carmine or Bauer-Feulgen methods, some sections should first be treated with saliva, then stained along with the others for comparison.

After the slides have been brought down to distilled water, cover the sections with saliva (spit on them) and allow them to stand from 15 minutes to ½ hour, changing the saliva several times. By this time all glycogen will be changed into sugar. Then wash very thoroly in water of about body temperature, as any mucus remaining will take up the stain and cause trouble later on. When counter-

[1]Grübler product used by the writer.

stained one can see at once where glycogen has been removed and whether or not other pink or red-stained materials are present.

Comparison of Methods

It requires exceptional care and considerable experience to make fully reliable glycogen preparations. If the particular glycogen in a tissue is especially labile and it is important to demonstrate all the glycogen present at a given time, then precautions must be taken to fix it immediately and uniformly in strong alcoholic solution (see p. 48). If plasmolysis of the intracellular glycogen is to be reduced to a minimum, fixation in aqueous formol-chrom-sublimate (Zenker-Bensley) or a similar one is indicated, but the chances are then against all the glycogen being preserved. If there are chrom-salts in the fixative and intracellular glycogen is to be studied, then the Best's carmine method must be used for staining because the iodine method is unsatisfactory for high-power work and the Bauer-Feulgen method does not stain glycogen specifically under these conditions. In alcohol fixed material all three methods are equally reliable. The Bauer-Feulgen method gives sharper microscopic pictures, small glycogen granules are easier to see, and the magenta color always distinguishes it from pink or red stained mucins or the like.

It is well to remember that these are staining methods and not microchemical tests and before we can speak with certainty as to the glycogen content of a tissue, it is necessary to make a chemical determination of glycogen. Where accurate quantitative data are needed, such determinations are essential.

Conclusion: There are, of course, advantages and disadvantages to the Best's carmine, Iodine and Bauer-Feulgen methods, and while one may become fairly expert at distinguishing glycogen from other cell constituents, it is never safe to judge a material of unknown glycogen content without some, and preferably all the controls possible, and to use several of the methods for comparison with each other. If one can fix one's own material promptly, however, in alcohol or formol-alcohol, the Bauer method is by all odds the one to use for beauty of color contrast, facility of study and accuracy of interpretation.

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THE USE OF JANSSENS' IRON HEMATOXYLIN IN PLACE OF THE WEIGERT ACID IRON CHLORIDE HEMATOXYLIN

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Some time ago the junior author, in search for a more satisfactory iron hematoxylin stain for tissue cultures, tried the old Janssens'[1] formula for an iron hematoxylin which Janssens called "hématoxyline noire," and which he described as having given "dans certaines circonstances des colorations d'une électivité tout à fait remarquable." It was found that this solution stained in 4 or 5 minutes, did not overstain in 30 minutes, and that a sample 4 weeks old was quite satisfactory. In this study the amount of ferric ammonium sulfate in the solution was reduced, from a saturated solution (in excess of 120 g. per 100 cc.) as recommended by Janssens, to 40 g. per 100 cc.

As we were using an iron hematoxylin and picro-fuchsin as a routine stain in the pathology laboratory we decided to give this iron hematoxylin formula a more extended trial. First a 160 cc. lot of Weigert's hydrochloric acid iron chloride hematoxylin, the formula previously in routine use, was made up and a record kept of the number of slides stained. This was used from June 18 thru June 25, and a total of 444 slides were stained. Those stained on the last day showed weakening of the nuclear staining and the solution was no longer considered satisfactory. Our usual experience, as well as that of others, has been that the Weigert formula is good for 7 or 8 days only.

We then prepared on June 20 a batch of 320 cc. of Janssens' formula from the same lot of hematoxylin but with the amount of alum further reduced, as follows:—

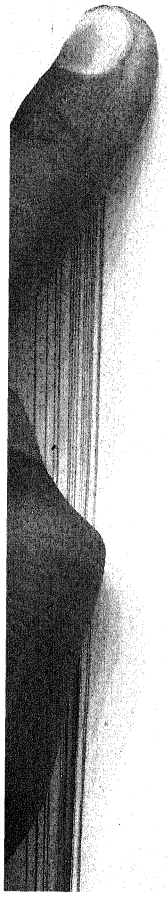
2 g. hematoxylin (Biosol, lot No. 84) was dissolved in 60 cc. absolute methyl alcohol (neutral, acetone free). This was added to a solution of 20 g. ferric ammonium sulfate (iron alum, violet crystals) dissolved in 200 cc. distilled water. To the whole, 60 cc. of C. P. glycerin were added.

The mixture was a deep blue-violet when freshly prepared and stained more intensely than when used later. In about an hour it

[1]Janssens, F. A. and Leblanc, A. 1898. Recherches cytologiques sur la cellule de levure. La Cellule, **14**, 203–9. (Year incorrectly given as 1897 in Lee's Microtomists Vade Mecum, 9 ed., p. 154, and 10 ed., p. 155.)

assumed a brownish black color and thereafter its staining properties were quite constant. Half of this solution was placed in the Stender staining jar, and to compensate for evaporation and carry-over two replenishments of 80 cc. each were made on July 5 and 13. The stain was used almost daily for 5 weeks, during which time a total of 1175 slides were stained. It was discarded July 25 as the nuclear stain showed some weakening.

Janssens' iron hematoxylin is considered, therefore, to be stable for 4 or 5 weeks, as compared with 1 week for Weigert's; it gives indistinguishable staining from *fresh* Weigert's iron hematoxylin, stains satisfactorily in 5 minutes, does not overstain in 30 minutes, and when the greater stability of the solution is considered, consumes less hematoxylin.



THE PREPARATION OF HARDENED EMBEDDING PARAFFINS HAVING LOW MELTING POINTS

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ABSTRACT.—Technical "stearic acid" hardens paraffins melting at 52° C. and above, and at the same time lowers the melting point. Spermaceti wax further lowers the melting point of such a mixture without much effect on the hardness. With these two substances, and one of the anti-crystallizing adjuvants already found satisfactory, embedding media yielding thin sections at room temperature and having melting point below 52° C. can easily be prepared. A general method is given, specific formulas are stated, and the behavior at various temperatures of typical embedding media of this kind is described.

For the safe infiltration of delicate material, the embedding paraffin should have a melting point not higher than 52° C., and for the best results in cutting 3 μ and thinner sections from material embedded in an ordinary embedding paraffin having such a melting point, the room temperature must be from 0° to −2° C. (Chamberlain, 1932). Otherwise, apparatus and tissue must be chilled with ice. An embedding paraffin having the required low melting point, but hard enough at the normal room temperature to yield thin sections without cooling devices of any kind, would obviously be much more convenient. If the cutting of thin sections at normal laboratory temperatures could be made possible, it also seemed that the cutting temperature of the paraffin block would be more nearly uniform, stable, and reproducible. Furthermore, ribbons of such a hardened paraffin would not become limp and sticky after leaving the knife, since they would not have to be brought into contact with an atmosphere of a temperature higher than that at which they were cut. The writer has, therefore, studied the properties of numerous waxes and other substances with a view to modifying the relationship of the melting points of paraffins to their room temperature hardness.

The best results, thus far, have been obtained with *technical "stearic acid"* (the "stearin" of commercial candle-stock mixtures), supplemented by smaller additions of *spermaceti wax*. With use of these two readily available modifiers and a suitable anti-crystallizing adjuvant it became possible to prepare from the common commercial

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paraffins embedding media melting at temperatures below 52° C. and hard enough at a temperature between 23° and 24° C. (74° F.) to permit the cutting of good 3 μ and fairly good 2 μ sections. At about 71° F., 1 μ sections were cut (Table 2).

Technical "stearic acid" (stearic acid containing palmitic acid and small percentages of other fatty acids) did not, at first, seem very promising for use in embedding media because it has a high melting point (55°–60° C.) and must be used in rather high percentages. As a matter of fact, however, stearic acid does not raise the melting point of an ordinary paraffin but actually *lowers* it (probably because the two substances are not of an isomorphous crystalline structure) to an extent depending upon the grade of stearic acid used and increasing with the percentage added up to more than 25%. Geller (1935) reproduces part of a melting-point table prepared by Lüdecke (1926, p. 108) to show the effect of from 2–25% of various waxes and other substances on the melting point of a paraffin, melting in the pure state at 52° C. According to these data, 14% of a stearic acid preparation melting at 55° C. lowered the m. p. of this paraffin to 50° C., and the 25% mixture had a 49.2° C. m. p. In the writer's experiments with the "triple-pressed" grade, from 16–20% of technical stearic acid hardened very effectively, and the larger percentages reduced the m. p. of the common commercial paraffins (m. p. about 54°–55° C.) to about 52°. Further lowering by stearic acid alone, however, could only be had by carrying the hardening to such a point that only the thinner sections (5 μ or less) could be cut at normal room temperatures without excessive curling. A lower m. p. with less extreme hardening can be had by starting with a slightly softer paraffin base. In the writer's work on these mixtures, however, a better modification in this direction has thus far been obtained by including a small proportion of spermaceti wax with the stearic acid.

Spermaceti wax (m. p. 42°–47° C.) was suggested by the late G. L. Bidwell[2] as another possible hardener, as it is harder than paraffin and has so low a m. p. that it should lower the m. p. of the mixture. This wax proved, however, to have the more valuable property of lowering still further the m. p. of paraffins to which stearic acid had been added, without appreciable effect in either direction upon the hardness conferred by the stearic acid. The hardness may, therefore, be adjusted, by means of the stearic acid, to suit both the expected laboratory temperature (winter or summer) and the range of section thickness desired; and, within certain limits, any further reduction

[2]Food Control Division, U. S. Dept. Agric., Food and Drug Administration, Washington, D. C.

of m. p. which the nature of the paraffin base may require can then be secured by means of a suitable percentage of spermaceti. The limiting proportion of this wax seems to be about 7%. Larger percentages began to lessen the cohesiveness and tenacity of the paraffin. On the other hand, as little as 1% of spermaceti increased distinctly the rate, relative to the percentage of stearic acid added, at which the latter modifier lowered the m. p. of the paraffin.

Anti-crystallizing adjuvants have already been proposed by several investigators. Their effects do not seem to be appreciably modified by the presence of technical stearic acid and spermaceti. Johnson (1903) added 1% of crude rubber, with a trace of asphalt. Keeping the paraffin melted for a long time, however, caused a part of the rubber to precipitate. McClung (1929) gives Johnson's formula, and adds that small quantities of castor oil may be effective. Chamberlain (1932) finds that bayberry "wax" (a vegetable tallow, or hard fat, not a true wax) "is likely to improve any paraffin except the best." The quantity specified would amount, by weight, to about 0.1%. The same author notes that Land found asphalt very effective. Waddington and Kriebel (1935) added 0.5% of a "petroleum ceresin," more fully described by Higgs (1935) with special reference to the modification of industrial paraffins. This proportion (0.5%) of "petroleum ceresin" slightly *raised* the m. p. of the paraffin. Higgs, however, found as little as 0.3% sufficient to produce the desired "microcrystalline" condition in paraffins, and this smaller proportion did not affect the setting point. It should be noted that "petroleum ceresin" is quite distinct from the ceresin prepared by refining ozokerite, and also from any form of paraffin.

To this list the writer can add Japan "wax" (like bayberry wax, it is actually a vegetable tallow), which has an effect practically indistinguishable from that of bayberry wax; the chloroform-soluble components of hard coal tar (suggested by Land's use of asphalt) when freed from solvent by prolonged heating; and bleached ozokerite or white ceresin.[3] Of the tar preparation, as little as 0.05% was effective, and did not change the m. p. Like asphaltum, however, it caused a marked dull yellow coloration. The ozokerite preparation, m. p. 72° C., checked crystallization at a concentration of 0.1%. At 0.5% it slightly raised the m. p.

The paraffin base: The cutting properties of the unmodified paraffin seem to have little or no influence on those of the embedding medium

[3]The writer is indebted to Dr. George S. Jamieson, Bureau of Chemistry and Soils, U. S. Dept. Agric. for the samples of ozokerite ceresin and candelilla wax used in the experiments described, and for much information on waxes, vegetable tallows, and related substances.

obtained by combining it with the adjuvants here considered. The common commercial paraffins, tho no one of them is a good embedding medium *per se*, have given quite as good results in the preparation of stearic-spermaceti paraffins as have the paraffins sold especially for biological work. Paraffins from the western "crudes" are said to have higher melting points than have those from the eastern oils.[4] The writer found the m. p. of "Texwax" more than 1° C. higher than that of "NJ Parowax." The latter paraffin thus far has given slightly better results in stearic-spermaceti mixtures than has any other paraffin base, either a biological or a commercial paraffin.

Soft paraffins (m. p. about 45° C.) are not suitable for hardening with stearic acid. Their melting points are not lowered but raised by the hardener, and good cutting properties are not produced. A minor proportion of such a soft paraffin added to a hard paraffin (m. p. 50°–55° C.) may, for some purposes, give better results than the hard paraffin alone. (See formula SSB 2, described below.)

The preparation of stearic-spermaceti paraffins: Except for occasional attention to funnel temperature during filtration, the preparation of the modified paraffins requires but a few minutes. The paraffin base is melted and held at a temperature of from 60°–70° C., the stearic acid ("triple-pressed" grade) and spermaceti wax are added, together with from 1–2% of either bayberry or Japan wax (tallow). These components are mixed thoroly and the completed embedding medium is poured into a hardened filter paper in a hot-water-jacketted funnel. In a funnel large enough to take 0.5 Kg. of the paraffin at once this quantity passes thru a hardened paper in about 40 minutes.

The thin grayish crust found on the surface of some lumps of bayberry wax does not dissolve in melted paraffin and is difficult to filter out. This coating should be completely removed before the lump is cut for weighing. Japan wax causes a slight opalescence which does not filter out. In the writer's experiments thus far, however, this has been so faint as to be harmless.

Formulas and behavior of some typical SSB (stearic-spermaceti-bayberry) paraffins: The paraffins described below have all given good results at the temperature and section thickness specified. Their formulae should, however, be regarded as illustrative only. The general method is obviously adaptable, by alteration in the percentage of the hardener, both to higher or lower cutting temperatures and to other section thickness.

Melting points were determined by heating very slowly 3 g. samples, in which the thermometer bulb was immersed, until the en-

[4]Information furnished by Dr. G. S. Jamieson.

tire sample melted to a clear, thin liquid. Smaller samples gave results lower than those observed in the paraffin oven. Cutting tests were made on a rotary microtome. The blocks were cut 1 cm. square; the block, microtome, and knife being brought to room temperature before each test. To secure absolutely uniform conditions of comparison, the data of Table 2 were obtained from blocks of the same size and without any embedded material. These paraffins have also been tested practically in the cutting of similar sections of *Vicia faba* root tips.

The formulae of the following paraffins are shown in Table 1.

TABLE 1. COMPOSITION OF STEARIC-SPERMACETI PARAFFINS 1–4.

Formula Number	Techn. Stearic Acid	Sperma-ceti Wax	Bayberry "Wax"	Paraffin Base	Nature of Paraffin Base
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	
SSB 1	16.	3.	1.	80.	"NJ Parowax"
SSB 2	16.	3.	1.	80.	70% "NJ Parowax"+30% soft paraffin (45° m.p.)
SSB 3	25.	1.	1.	73.	"Texwax"
SSB 4	25.	1.	1.	73.	"NJ Parowax"

Paraffin SSB 1 melted to a clear, thin liquid at 51.3° c., which was 3.2° below the m. p. of the pure paraffin from which it was made. By operating the microtome slowly, smooth 7 μ sections were cut at 86° F. At 83° F., with slow cutting, the 3 μ sections were fairly good, the 4 μ sections good, and the 5 μ sections very good. These 5 μ sections expanded much less than 10% on the slide (cf. Chamberlain's criteria, footnote under Table 2) and showed only a slight permanent compression. At 74° F., equally good 3 μ sections (*Vicia faba* root tips) were cut. At 69° F. the 3 μ sections were still more crisp, were slightly bowed unless the microtome was run rather briskly, and broke apart readily when long ribbons were cut. For further data on 3 μ and 2 μ sections, see Table 2. Satisfactory 1 μ sections were not obtainable from SSB 1 at normal room temperatures.

Paraffin SSB 2 was made from a paraffin base (m. p. 51.9° C.) consisting of a 3:7 mixture of 45° C. paraffin with "NJ Parowax." The m. p. of this embedding medium (49.6° C.), was the lowest thus far obtained in a paraffin yielding thin sections at normal temperatures. The behavior of this paraffin at 69° F. is shown in Table 2. At 79° F. it yielded (at a very slow cutting rate) good 5 μ and 4 μ sections, and fair 3 μ sections. The 2 μ sections were poor at this temperature, however. For the infiltration of material easily injured by heat SSB 2 is the best of the formulae here given, but it obviously does not stand the test of high summer temperatures as well as does SSB 1.

Paraffins SSB 3 and SSB 4 were made up to determine if the hardness could be raised to a point at which 1 μ sections could be cut at ordinary room temperatures without loss of other essential properties. "Texwax" has a melting point (55.6° C. in the case of the samples tested by the writer) higher than that of "NJ Parowax" (54.5° C.), and it therefore seemed quite possible that the "Texwax" preparation (SSB 3) might be harder than that made from "NJ Parowax" (SSB 4). The reverse was the case, as is shown in Table 2. Both yielded 1 μ sections, but the paraffin base of the lower m. p. gave in this case an SSB preparation of definitely better hardness.

TABLE 2. BAHAVIOR OF CERTAIN SSB PARAFFINS AFTER STANDING 18 HOURS IN A TEMPERATURE-CONTROLLED ROOM AT FROM 69°–71° F.*

Referance No. of paraffin	Melting point °C.	Cutting temperature °F.	Section thickness	Expansion on warming†	Fraction of full width recovered
SSB 1	51.3° C.	71° F.	3 μ	3.5%	0.92
SSB 1	51.3° C.	71° F.	2 μ	4.2%	0.84
SSB 2	49.6° C.	69° F.	3 μ	5.3%	0.85
SSB 2	49.6° C.	69° F.	2 μ	5.9%	0.80
SSB 3	51.0° C.	71° F.	1 μ	12. %	0.65
SSB 4	50.6° C.	71° F.	1 μ	11. %	0.71

*The wrinkling and permanent compression data here shown refer to sections cu at a rate much slower than is customary in the use of a rotary microtome. Except in the handling of long ribbons, a good sliding microtome might be more suitable for the cutting rate needed for the 2 μ and 1 μ sections.

†Compare Chamberlain's criteria (1932, p. 126) for 5 μ and 1 μ sections: "If a 5 μ ribbon does not lengthen when warmed on the flooded slide more than 5%, the cutting may be regarded as good; at 1 μ, if it does not lengthen more than 20% the cutting has been good."

Advantages and Disadvantages: The convenience of the hardened paraffins is obvious. The desirability of an approximately reproducible cutting temperature has already been noted, together with the probability of better behavior of the hardened ribbons in warm air. To test the heat tolerance of such ribbons, about 20 cm. of a ribbon 3 μ thick (SSB 1) was left on dry paper thru two days of warm weather (maximum about 88° F.). At the end of this time the entire ribbon could still be picked up intact.

A test of one of these hardened paraffins was made by Dr. M. N. Pope[5] who found, as disadvantages of this type of embedding medium, that the blocks are not as transparent as are those of a pure paraffin; and that great care had to be used in flattening the ribbons on the

[5]Of the Bur. of Plant Industry, U. S. Dept. of Agriculture, Washington, D. C. The writer is also indebted to Dr. Pope for the opportunity to use a Spencer rotary microtome in obtaining the data given in Table 2; and for the suggestion to measure and include in this table the data given in the last column.

slide, since too much warmth set free the sections from the paraffin. Advantages noted by Dr. Pope were that slower solidification occurs than in the case of pure paraffins; that there was little deformation; and that the ribbon handled well at 89°–90° F., sticking neither to the instruments nor to the fingers at this temperature.

Like all hard paraffins, the stearic-spermaceti hardened media do not ribbon readily when the temperature is a little lower than that best suited to the thickness of the sections. A hot needle, dipped into soft paraffin, may be applied to the upper and lower surfaces of the block (Chamberlain, 1932, p. 125). The writer, however, prefers the heated end of a slide. A single touch with the slide covers the whole surface with a thin and even coat of the softer paraffin. In using the hardened paraffins here described, this treatment may well be given every block as soon as it has been mounted on its wooden support. This will avoid an unknown change of temperature after the block has come to room temperature and is ready for cutting. For the same purpose—that the room temperature may correctly represent the actual cutting temperature—the writer avoids touching either the paraffin or the knife with his fingers in attaching the paraffin block to the microtome. If these two precuations are taken, the degree of hardening best adapted to the prevailing laboratory temperature and the required section thickness can easily be determined and thereafter adhered to.

The *solubilities* of the stearic-spermaceti paraffins seem to be essentially the same as those of pure paraffins. This statement applies specifically to solubility in the benzene hydrocarbons; in chloroform and carbon tetrachloride; in the butyl alcohols; and in *p*-dioxan. The stearic-spermaceti paraffins show much less tendency to separate in long, acicular crystals from saturated solutions in benzene, toluene, or xylene than do the pure paraffins. A previous article has already described (Waterman, 1934, p. 28) how the writer has utilized the re-solution, on warming, of suspensions of paraffin in the butyl alcohols to secure a gradual increase in the concentration of the paraffin solution to which the material is exposed before oven infiltration. The difficulty met with by McWhorter and Weier (1936) in securing complete infiltration after dioxan has been encountered by the writer after tertiary butyl alcohol and after dioxan. This was overcome by following the McWhorter and Weier method, consisting in the addition of a little xylene in two steps preceding oven infiltration.

Higgs' (1935) observations on the effect of small quantities of "petroleum ceresin" on paraffins would seem to make it almost certain

that this substance will prove superior to either bayberry or Japan wax if a commercial source of "petroleum ceresin" can be found.

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A NILE BLUE CULTURE MEDIUM FOR LIPOLYTIC MICROORGANISMS[1]

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ABSTRACT.—A culture medium for the enumeration of lipolytic microörganisms is presented which makes use of Nile blue sulfate as a specific indicator for detecting changes in neutral fats. It is prepared as follows:—20 g. agar (Bacto) is dissolved in 1000 cc. beef infusion broth prepared in the usual way; the whole filtered and the reaction adjusted to pH 7.4; 100 cc. amounts are distributed in flasks and sterilized in the autoclave at 15 pounds pressure for 20 minutes. A dye-fat emulsion is then prepared by first dissolving 4 g. ossein gelatin (200 Bloom, isoelectric point at pH 5.6) in 100 cc. distilled water at 60° C. and sufficient N/10 NaOH added to bring the reaction to pH 7.2. The gelatin solution is first shaken vigorously in a stoppered bottle which contains 100 cc. U.S.P. cotton-seed oil to which has been added 0.1 g. Nile blue sulfate (C.I. No. 913, dye content 90%, solubility 0.15 g. in 100 cc. water) and then put thru a hand homogenizer until the oil globules are about 10μ in diameter. After sterilization in the autoclave at 15 pounds pressure for 20 minutes, the emulsion which was originally blue, turns deep pink.

When plates are to be poured, 100 cc. of the sterile infusion agar base are liquified and while still hot, 10 cc. of the sterile dye-fat emulsion are added aseptically. The whole is well mixed and allowed to cool to 45° C. before being poured into plates. Before inoculation the medium has a rich salmon-pink color. Colonies of organisms which decompose fats appear as light blue colonies surrounded by a narrow zone of decolorized, transparent medium when incubated for 3-4 days at room temperature. Non-lipolytic colonies appear white or pink. The medium is equally efficient both with pour and steak plates, the former being desirable for quantitative work.

INTRODUCTION

During an investigation[2] undertaken for the purpose of establishing

[1]The author wishes to thank Dr. Louis Gershenfeld, Director of the Bacteriological Laboratories of the Philadelphia College of Pharmacy and Science, for the criticisms offered by him during the course of this investigation.

[2]Details of investigation submitted as thesis for the degree of Doctor of Science in Bacteriology at the Philadelphia College of Pharmacy and Science.

a relationship between the chemical changes that occur during the anaerobic decomposition of sewage sludge and the various microörganisms present therein, the necessity arose for making quantitative determinations of organisms possessing lipoclastic properties. After many unsuccessful attempts had been made to prepare and utilize several media which had been advocated for this purpose, the culture medium reported here was formulated.

Historical

Since Nencki's (1886) classic work on microbial lipolysis in the intestinal tract, a variety of culture media have been suggested for demonstrating the presence of fat-splitting microörganisms. In most instances, the proposed media employed one of the following for detecting changes in the fat: (a) indicators such as litmus and brom phenol blue for noting changes in acidity, Buchanan (1921), Waksman and Daviston (1926), Sayer, Rahn, and Farrand (1908) and Jensen and Grettie (1933); (b) production of colored soaps thru the use of copper sulfate and other metallic salts uniting with free fatty acids, Carnot and Mauban (1918) and Berry (1933); or, (c) specific indicators such as Nile blue sulfate, for free fatty acids, Turner (1929) and Smith (1907).

Jensen and Grettie (1937) made a comprehensive study of those media which employed the above and tho unable to find one entirely satisfactory, they reported that Turner's medium in which Nile blue sulfate was used, was superior to all other media investigated by them. Other evidence of the superiority of Nile blue sulfate as a specific indicator for detecting lipoclastic changes may be found in the work of Hammer and Collins (1934).

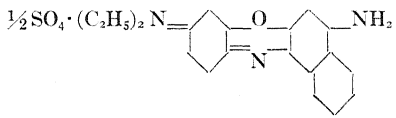
Considerable controversy has arisen regarding the kind of fat which should be utilized in preparing the fat emulsion employed in the medium. Turner (1929) employed cotton-seed oil. Jensen and Grettie (1937) who recommended and used cocoanut oil thought that cotton-seed oil, because of its high active oxygen content, inhibited the growth of many microörganisms. On the other hand, Hammer and Collins (1934) state that butter fat, beef tallow, cotton-seed oil and many other natural fats may be used with equally satisfactory results.

The media of Turner, of Jensen and Grettie, and of Hammer and Collins, were formulated by the respective authors primarily for quantitative purposes. Attempts by the writer to prepare these media satisfactorily were unsuccessful. This was due to the fact that sufficient data concerning the source and quality of the ingredi-

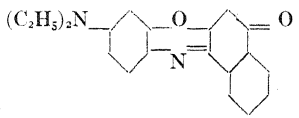
ents, especially of the Nile blue sulfate and the method for preparing the fat emulsion, were not given in the original publications.

Action of Nile Blue Sulfate on Fat

According to Conn (1936), Nile blue sulfate is a dye of the quinone-imine group. Its property of differential staining was first observed by Smith (1907) in studies on the staining of pathological sections. He observed that certain dyes of the oxazine series colored fat globules red while the protein matter was stained blue. Thorpe (1907) investigated this phenomenon and finally concluded that only dyes which are derivatives of phenonaphthoxazine yielded a red coloring matter that possessed a marked affinity for neutral fats. He also found that those derivatives containing an amino or substituted amino radical in position 6 exhibited this property to a greater degree. According to Thorpe, the action of water or very dilute mineral acid on Nile blue sulfate causes hydrolysis of the amino group in position 6 resulting in the formation of the corresponding oxazone as shown below.



Oxazine



Oxazone

The extent to which this hydrolysis takes place depends upon the temperature and the length of time during which the acid is allowed to act; but a condition of equilibrium between the salts of the two bases appears to be reached after boiling with water or dilute acid for 15 minutes.

The salts of both the oxazine and oxazone bases are blue and there is consequently no apparent change in the color of the dye solution after hydrolysis. The salt of the weaker oxazone base is, however, more readily dissociated and can be extracted from admixture with the oxazine by shaking with xylene, ether, or neutral fat. Accordingly, when a pathological section is stained in a boiled, aqueous solution of Nile blue sulfate, the protein matter is colored blue by the unchanged oxazine. The neutral fat globules, on the other hand, extract the oxazone base from the solution of its salt and are stained red.

It is evident from the foregoing that Nile blue sulfate is water soluble and in aqueous solutions possesses a blue color. When it is converted to the oxazone and the free base extracted, it imparts a red color to neutral fats in which it is soluble.

When a solution of the free oxazone in oil is mixed with a fatty acid, such as oleic acid, the red color is changed to blue. This reaction is specific and forms the basis of the following medium.

Preparation of the Medium

(a) Dissolve 20 g. agar (Bacto) in 1000 cc. of beef infusion broth prepared according to the directions of Zinsser and Bayne-Jones (1935). Filter thru absorbent cotton and adjust the reaction to pH 7.4. Distribute in 100 cc. amounts in flasks and sterilize in the autoclave at 15 pounds pressure for 20 minutes.

(b) *Dye-fat Emulsion:* Dissolve 4 g. ossein gelatin (200 Bloom, isoelectric point at pH 5.6) in 100 cc. of distilled water at 60° C. Add sufficient *N*/10 NaOH solution to raise the pH to 7.2. The gelatin solution, while still warm, is shaken vigorously in a large stoppered bottle with 100 cc. of cotton-seed oil (U.S.P.) to which has been added 0.1 g. Nile blue sulfate (C.I. No. 913, dye content 90%, solubility 0.15 g. in 100 cc. water)[3]. The mixture is then put thru a hand homogenizer until the oil globules are about 10 μ in diameter. This emulsion has a light blue color and after sterilization in the autoclave at 15 pounds pressure for 20 minutes turns deep pink. At room temperature the emulsion soldifies, but by placing it in the incubator at 37° C. a day before it is to be used no difficulty in pipetting is experienced.

(c) *Preparation of Plates:* When plates are to be poured 100 cc. of the sterile infusion agar base are liquified, and while still hot 10 cc. of the sterile dye-fat emulsion are added aseptically. The whole is well mixed and allowed to cool to 45° C. before being poured into plates. Before inoculation the medium has a rich salmon-pink color.

The medium is equally efficient both with pour and streak plates, the former being desirable for quantitative work. Colonies of organisms which decompose fats appear as light blue colonies surrounded by a narrow zone of decolorized, transparent medium when incubated for 3–4 days at room temperature. Non-lipolytic colonies appear white or pink.

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[3]The sample employed by the writer was purchased from Hartman-Leddon Co., Philadelphia, Penna.

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A NEW SHARPENING BACK AND PROCEDURE OF SHARPENING FOR MICROTOME KNIVES

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THE KNIFE BACK

In the course of several years' histological research, the writers have had constant concern with the sharpening of microtome knives. During this time they have devised a new sharpening back (patent pending) and a modification of the usual procedure in hand sharpening which they believe to be of sufficient value to warrant inspection and criticism.

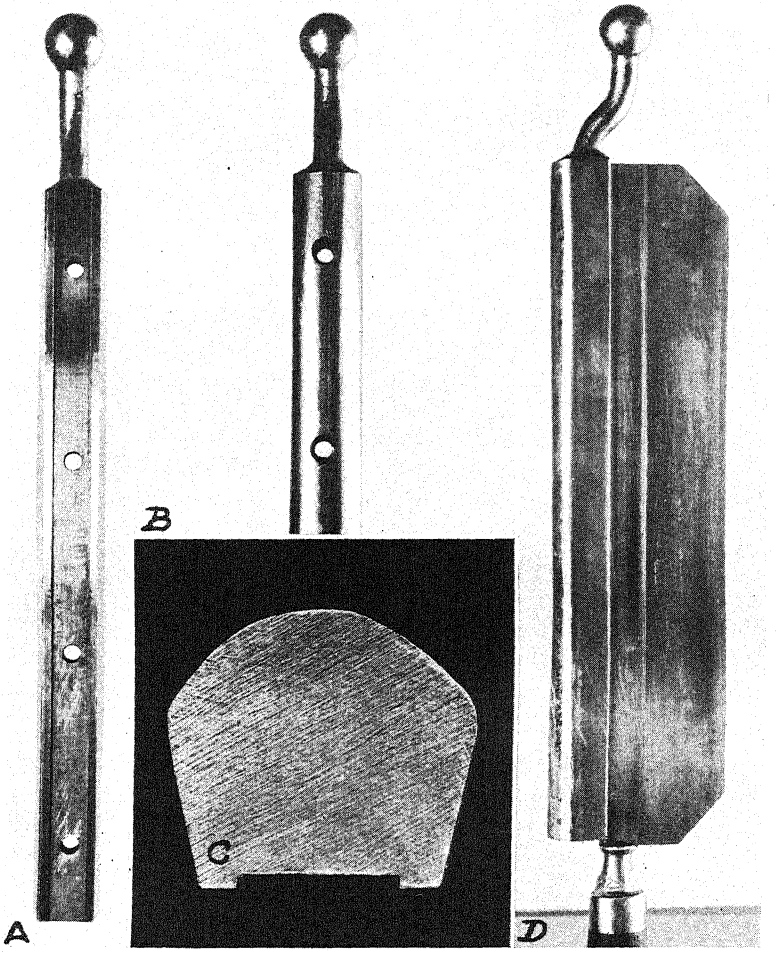
The new detachable knife sharpening back is probably sufficiently illustrated and explained by the accompanying plate. The principal advantages claimed for this back are: it is exactly replaceable in one position; it is immovable in use and has long wearing qualities. Two such sharpening backs have been made and during a year's trial have thoroly fulfilled expectations.

THE SHARPENING PROCEDURE

In describing their sharpening procedure, the writers will, of course, attempt to give only supplements to the general method. They also wish to state that their modification of general sharpening procedure in no way represents their belief that it is the only efficient method. Rather, it is their practical statement of conclusions reached after considerable experimentation.

THE HONE: A combination grit carborundum stone, $8 \times 2 \times 1$ inches (No. 108 made by the Carborundum Co., Niagara Falls, New York) has been found most satisfactory. (In the writers' opinion this length of stone is quite sufficient for a 180 mm. knife.) Previous to use, a new stone should have all edges slightly beveled to prevent chipping. In use the stone should rest on two or three thicknesses of wet cardboard which serve to hold the stone in place and provide a slight "give" as a preventative against injury to the knife edge. Distilled water is used as a lubricant in honing. The usual correct treatment of the stone in honing procedure should, of course, be practiced.

It has been found to be a distinct advantage to grind away the heel and toe corners of the cutting edge (shown in D of the accompanying



DETACHABLE SHARPENING BACK FOR MICROTOME KNIVES

(A) The sharpening back with view of channeled side.
(B) Reverse side of (A) showing rounded back and countersinking for attaching screws.
(C) An enlarged cross-section view of the sharpening back. The outline represents the rounded back at top, the grinding faces at the sides, and the channel below.
(D) The assembled sharpening back and knife ready for honing. (Note cutting down of knife blade at heel and toe to aid in honing.)

plate). This greatly facilitates honing at the knife ends and thereby aids in keeping a straight edge. Knives which have been ground down so that the cutting facets are wider than 4 or 5 mm. may (using care) be hollowed out with a power grinder so that the cutting facets

are narrowed to 2 or 3 mm. Slightly varying width of the cutting facets seems to have no ill effect on the knife's cutting qualities if the edge is straight and the angle between the cutting facets is approximately the same at all places along the blade. In preparation for honing, the knife and knife-sharpening back should be carefully cleaned—particularly the back of the knife blade and the channel of the sharpening back so that an exact union is made.

The honing operation: The usual heel to toe diagonal stroke is used and the progress of the grinding on both cutting facets should be observed under a low power microscope. Such examination enables the operator to judge his faults in honing and the completion of grinding. One of the common difficulties in hand honing is the production of a bowed edge. Regular honing at either end of the blade will greatly obviate this. Short strokes of the same diagonal as is used in full length honing should be made with occasional full length strokes to join the grindings smoothly. With correct technic the depression at the blade center may be easily kept within 1 mm. in 150 mm. of knife edge.

First stropping: This is done on a length of 3-inch canvas belting laid over a $2 \times 3 \times 15$ inch piece of wood and tacked over at the ends. Tripoli powder wet with water is used as an abrasive. The "honing" stroke is the same as for leather stropping—toe to heel, back foremost. The sharpening back is kept in place and both hands used as in honing. The progress of the polishing is also watched under the low power microscope.

Second stropping: The stropping is completed on leather. Care should be taken to keep the strop taut to prevent excessive "rounding" of the edge. The writers judge that when the knife will clip a human hair at a distance of 1 cm. from the hand the knife is ready for average sectioning purposes.

Acknowledgment is made to the Works Progress Administration for technical assistance under O. P. 465-94-3-39.

LABORATORY HINTS

FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

J. A. de Tomasi — *Abstract Editor*

MICROSCOPE AND OTHER APPARATUS

ZDANSKY, O. **An improved apparatus for the serial sectioning of fossils.** *Science*, **88**, 385–6. 1938.

The modifications here suggested apply to Simpson's holder by making it more sturdy and increasing its accuracy. The number of parts is smaller and its design is kept within the range of an average mechanic's skill. It consists essentially of a holder carrying a millimeter scale above the thread. A sleeve rotates around it with divisions on its conical upper end and the 2 scales afford micrometer readings to 0.01 mm. Advantages claimed are: There is no delicate, easily bent pointer; the threads are protected; the scale is an integral part of sleeve; and mistakes in taking readings are less probable.—*J. A. de Tomasi.*

PHOTOMICROGRAPHY

PIJPER, A. **Dark-ground studies of flagellar and somatic agglutination of B. typhosus.** *J. Path. & Bact.*, **47**, 1–17. 1938.

The author finds sunlight superior to artificial light, including a 20 amp. arc lamp, for observation and photography of flagella of living bacteria. The source of sunlight is a Zeiss heliostat mounted on an adjustable glass plate. A collecting lens of 2 meter focus stands between the heliostat mirror and the microscope condenser. The microscope mirror is omitted. Coolers filled with Mohr's salt are indispensable. The other equipment includes a Zeiss cardioid condenser. New slides are used, and are prepared by dipping into a collodion solution. When the skin is nearly dry it is pulled away taking the dirt with it. Oil immersion apochromats 35× and 60× with 20× ocular make a useful combination for photography. Zeiss photographic equipment is used thruout: Contax camera with new "beam-splitter" developed for use with the Zeiss cinema camera.

The bacteria are suspended in a vaseline-sealed wet film preparation, one corner of which is left unsealed to allow diffusion of air.—*S. H. Hutner.*

MICROTECHNIC IN GENERAL

HAGAN, H. R. **Overcoming static when sectioning with the microtome.** *Teaching Biologist*, **8**, 59. 1939.

Trim the mounted block on its upper and lower sides, dip in melted soft paraffin, cool quickly, trim the ends of the block, and section. The soft paraffin will hold the sections together and prevent the ribbon from breaking so easily.—*Virgene W. Kavanagh.*

DYES AND THEIR BIOLOGICAL USES

CH'IN, T. L. **Influence of color filters in photodynamic action of fluorescent dyes on gonococcus.** *Proc. Soc. Exp. Biol. & Med.*, **38**, 697–700. 1938.

Various dilutions (10^{-1} to 10^{-10}) of eosin, methylene blue, trypaflavin, mercurochrome, protargol, argyrol, and dimethyl and tetramethyl paraphenylenediamine hydrochloride were made in dist. water. Suspensions in these dyes were exposed

to light, using Eastman Kodak filters red (F29), orange (G15), yellow (K-1,6) green (B58), blues (H45 and C49) and violet (D35). Samples were tested for viability after 5–120 minutes of exposure. The gonococcus was particularly sensitive, even more so than other Neisseria; control tests indicated survival in dye dilutions not treated with light. Trypaflavin and methylene blue were most effective with an orange filter and eosin with green and light blue filters. Organisms with dilute dyes appeared, in a general sense only, to be sensitive to selected wave lengths of light.—*M. S. Marshall.*

FIGGE, F. H. J. **Inhibition of tyrosinase melanin formation by sodium bezenone-indophenol.** *Proc. Soc. Exp. Biol. & Med.*, **39**, 569–71. 1938.

Sodium bezenone-indophenol prevents the formation of pigment granules, apparently by its action on tyrosinase. The pallor induced in young amphibian larvae by this and similar dyes may be explained on this basis.—*M. S. Marshall.*

GRAFFLIN, A. L. **The absorption of fluorescein from fresh water and salt water by Fundulus heteroclitus, as judged by a study of the kidney with the fluorescence microscope.** *J. Cellular & Comp. Physiol.*, **12**, 167. 1938.

Specimens of the euryhaline species, *Fundulus heteroclitus*, were exposed to a dilute solution of fluorescein in fresh water and sea water. The animals were killed after different periods of immersion, and the extent of absorption of the dye was estimated by a study of the kidney with the fluorescence microscope. Observations indicate that the dye is absorbed quite markedly from sea water but only in traces from fresh water. These results are interpreted in terms of the obligatory swallowing of sea water in marine teleosts, and absence of such swallowing in fresh water teleosts.—*L. Farber.*

IVANOV, M. F. and BRAUN, A. A. **On the problem of permeability of tissue membranes.** *Arch. Russ. Anat. Hist. Embriol.*, **19**, 161–74. 1938.

Striated muscle membranes were found best for studying the permeability of tissue membranes. Neutral red and cyanol were the most suitable dyes. Results obtained with these two dyes of opposite reaction are closely comparable. It is found that permeability of live membranes increases with time. In the case of a dead membrane, however, permeability is consistently lower up to the fifth hour, after which time its value increases faster than in the case of the live membrane. This is probably caused by a greater absorption of the dye by the dead membrane in the first few hours until saturation is reached, thus allowing greater, or faster, permeation. Temperature increases up to 28° C. enhance the permeability of the live membranes.—*J. A. de Tomasi.*

MAURER, F. W. **Absorption of acid indicator dyes by living frog muscle.** *J. Cellular & Comp. Physiol.*, **12**, 379. 1938.

Phenol red absorption by living muscles can be accounted for without involving any absorption by the muscle cells. Killed muscle cells absorbed large amounts of dye. Muscle cells made acid in death absorbed large amounts of dye, while muscle made alkaline in death absorbed little more than living muscles.—*L. Farber.*

SMOILOVSKAYA, E. J. **The supravital staining of surviving sections of normal tissues in physiological and hypotonic surroundings.** *Arch. Russ. Anat. Hist. Embriol.*, **19**, 105–15. 1938.

In this study neutral red is made up in concentrations varying from 1:200,000 to 1:50,000 in Ringer-Locke soln. With sections from such materials as live lung, kidney and liver from the common laboratory animals it is found that concentrations higher than 1:5,000 are toxic. In isotonic solutions, the dye accumulates promptly in the cytoplasm in epithelial cells, while in hypotonic solutions the nuclei are rather apt to take on the dye. This reversal of the reaction appears to be linked to the loss of phosphates from the tissues into the surrounding fluids.—*J. A. de Tomasi.*

STRELING, G. S. **On the physiological gradient. III. Vital stains reduction gradients in asphyxiation in Oligochaeta and their correlation with injuries.** *Arch. Russ. Anat. Hist. Embriol.*, **19**, 226–43. 1938.

Early experiments proved that certain indicators will cause injury to such worms as *Limnodrilus sp.* and *Tubifex tubifex*. This study is intended to find an interpretation of the oxidation-reduction gradients observed, using methylene blue, Janus green, thionin, and brilliant cresyl blue as vital stains and indicators. It is found that prolonged staining during asphyxiation injures the tissues more rapidly and tends to shorten the reduction time of the indicators. Furthermore, axial (total body length) as well as portional (partial lengths), and dorsoventral gradients are observed in the animals under study. Typical observations: the redox potential may be lowered by mechanical injury, by heat, and by the action of Janus green. It appears that the loss in potential can be correlated with an increase in adsorption by the tissue protoplasm, indicating a gain in stainability.—*J. A. de Tomasi.*

TENNENT, D. H. **Some problems in the study of photosensitization.** *Amer. Naturalist*, **72**, 97–109. 1938.

This work was undertaken to study the experimental changes induced in the cell division of eggs of the sea urchin by the combined action of dyes and sunlight. Out of 30 dyes used, results with neutral red, brilliant green, auramine O, acridin, eosin Y and picric acid are reported. In every instance irradiation with sunlight raised the effectiveness of dilute dye solutions to the level of results produced by strong solutions without irradiation. Every dye has its own characteristic effect. Irradiation in dye solution is far more effective when heat is part of the system. Photodynamic action is not proportional to the degree of fluorescence of a dye. The possibility of a photodynamic effect by photo-compounds of the irradiated dyes may be considered, but has not been proven.—*J. A. de Tomasi.*

T'UNG, T. **Intracutaneous immunization of rabbits with photodynamically inactivated Type I pneumococcus.** *Proc. Soc. Exp. Biol. & Med.*, **39**, 159–61. 1938.

Pneumococcus Type I, inactivated by methylene blue and light, was compared with formalin treated suspensions. Photodynamically treated suspensions are less susceptible to autolysis and are antigenically superior as judged by agglutination tests, precipitin tests with soluble specific substance, complement fixation tests and protection tests.—*M. S. Marshall.*

ANIMAL MICROTECHNIC

BLACK, E. **The staining reactions of fats after the use of various dyes and fixing agents.** *J. Lab. & Clin. Med.*, **23**, 1027–36. 1938.

Fat was injected intravenously into a white rat. After asphyxia occurred representative pieces of lung were placed in normal saline for fresh frozen sectioning; other pieces were fixed in various solutions, frozen, and sectioned. The technics of fixing and staining were those described by Warthin in Practical Pathology. Several tables are presented for convenience in identifying various fats fixed with different reagents. The fat dyes used were: Scharlach R, Sudan III, Nile blue sulfate, OsO_4, and indophenol. The author considers poppy seed oil, lipiodol, olive oil, lard, butter fat, mineral oil and paraffin. The fixatives used were formol, $HgCl_2$, and Müller's fluid. Within the limited group studied, vegetable oils stained somewhat more deeply than did animal fats.—*P. R. Beamer.*

COMMONER, B. **On the nature of the staining of unfertilized Chaetopterus eggs by neutral red.** *J. Cellular & Comp. Physiol.*, **12**, 171. 1938.

The uptake of neutral red by unfertilized *Chaetopterus* eggs was found to be a first order reaction, in which the staining rate is proportional to the amount of unstained, stainable material in the eggs. Within limits the staining rate was proportional to the outside neutral red concentration and was increased by a rise in temperature up to 20° C. Above this temperature stained eggs lose their

neutral red binding capacity and set free the bound stain. The rate of loss of neutral red at temperatures up to 43° C. was proportional to the rate of heat denaturation of the egg protein. It was concluded that the staining of the eggs is due to the combination of the stain and some protein constituents of the cells.—*L. Farber.*

COOPER, Z. K. and SCHIFF, A. **Mitotic rhythm in human epidermis.** *Proc. Soc. Exp. Biol. Med.*, **39**, 323–4. 1938.

To count the number of mitotic figures in the epidermis, drop the fresh skin into 1% acetic acid for 24 hr., separate epidermis from dermis and discard dermis, stain epidermis *in toto* in Ehrlich's hematoxylin, dehydrate in alcohol, clear in oil of wintergreen and xylene, mount.—*Virgene W. Kavanagh.*

DEBAISIEUX, P. **Organes scolopidiaux des pattes d'insectes.** *La Cellule*, **47**, 79–202. 1938.

Detachment of sections of chitinous insect parts from the slide during staining may be prevented by adding a temporary collodion film. After removing paraffin with xylene and passing sections thru abs. alcohol, immerse them in a solution of collodion in at least 4 vols. of abs. alcohol+ether; then immerse in 80% alcohol, hydrate and stain. After dehydrating the stained sections with abs. alcohol, dissolve collodion film in ether; then pass sections thru abs. alcohol and xylene and mount in balsam.—*L. W. Sharp.*

DEI POLI, G. and POMERRI, G. **Un metodo semplice e sicuro di colorazione della sostanza cromofila (zolle di Nissl) delle cellule nervose.** *Monit. Zool. Ital.*, **49**, No. 5, 123–4. 1938.

Fix a small piece of nervous tissue (removed as soon as possible after death) for 24 hr. or more in 95–98% alcohol, or in 10% formalin (in water or physiological salt solution). Dehydrate in alcohols, clear in xylene and embed in paraffin. Immerse in the following staining mixture for 3–4 min.: Dist. water, 100 cc.; carbol fuchsin (basic fuchsin, 0.2 g.; conc. phenol soln., 1.0 cc.; 95% alcohol, 2.0 cc., dist. water, 20.0 cc.), 2.5 cc.; glacial acetic acid, 0.5 cc. Wash rapidly in dist. water. Differentiate for 3–5 min. in the following mixture: dist. water 100.0 cc.; formalin, 1.0 cc.; glacial acetic acid, 1.0 cc. Wash sections in dist. water; dehydrate in a series of alcohols; clear in xylene; mount in neutral Canada balsam.

Sections of tissue fixed in formalin do not need as long a treatment with the formol-acetic acid mixture and should be washed several minutes in 95% alcohol until the surrounding tissue seems to be decolorized.

With this method the staining of the tigroid substance is uniformly good and controlled differentiation under the microscope is not necessary. The chromophile substance and nucleoli are stained sharp red; the nucleus and all other parts of cells and tissue remain colorless.—*A. B. Dawson.*

FRIELING. **Universal-methode für die Färbung von Bindegewebe und elastistischen Fasern.** *Mikrokosmos*, **31**, 82. 1938.

Solution A: Chrom-Hematoxylin "H" 2.00 g., tap water 100.00 ml.; dissolve on water bath 5–10 min., cool and filter. *Solution B:* Blochmann elastin stain "H" 0.5 g., 70% alcohol, 100.00 ml.; filter.

Staining Procedure: Deparaffinize sections; stain in Sol. A. 10–15 min.; rinse in water to remove excess stain; rinse in 70% alcohol; transfer to elastin stain (Sol. B); rinse in 95% alcohol; dehydrate; xylene; balsam.

Result: Nuclei black, collagen fibers blue, elastic fibers dark brown. *Note:* If red nuclei are desired, stain in Kernechtrot-aluminum sulfate for 5 min. after elastin stain. (Kernechtrot-aluminum sulfate "H" 2.00 g., hot water 100.00 ml.) —*J. M. Thuringer.*

HSÜ, F. **Étude cytologique et comparée sur les sensilla des Insectes.** *La Cellule*, **47**, 7–60. 1938.

A solution for injecting larvae of *Choaborus crystallinus* is made as follows: Dissolve 0.2 g. Grübler's methylene blue in 10 cc. dist. water. Add 4 drops of 25% HCl and then 0.4 g. rongalite white. Heat gently until clear. Filter if

necessary and store in stoppered bottle in the dark for a few days. The solution retains its efficacy for 2 weeks. Just before injection mix 1 vol. of the solution with 4 vols. of 0.75% NaCl. Inject 2–4 drops of the mixture into each larva. Fix in sat. soln. of ammonium molybdate.—*L. W. Sharp.*

LEACH, E. H. **A new stain for mucin.** *J. Path. & Bact.*, **47**, 637–9. 1938.

Within a few months, a sat. solution of Sudan black B in 50% diacetin hydrolyzes and loses its property of staining fats, but will stain mucin in both frozen and paraffin sections. The new stain shows greater stability and specificity. It may also be produced by hydrolysis of Sudan black B with acetic acid, forming a new dye "mucisudan".

Prepare the stain by adding 2 g. mucisudan to 100 cc. 0.5% HCl in 70% ethanol. Allow to stand at least two days on a hot plate; filter. A slightly less satisfactory solution is prepared by boiling, cooling, and filtering. Tissue is preferably fixed in Zenkerformalin. Before staining place the sections in acid alcohol 1 min., then in the stain solution 1 hr. Treat with acid alcohol a few minutes; counterstain in carmalum or neutral red. Dehydrate and differentiate in abs. alcohol a few minutes. Mucin, cartilage and occasionally elastic fibers stain dark brown; nuclei, red.

It is difficult to over-differentiate. If the differentiation period is shortened, the granules of mast cells are stained. Alcohol and all other reagents must be free of traces of other dyes. The stain solutions require occasional filtration.

Greatly improved preservation and staining of mucin is obtained if 96% alcohol is used instead of water for spreading. Smear slide with conc. soln. of glycerol and egg albumen. Flatten out sections on 96% alcohol, by warming slide on hot plate. Dry slide at 55° C. at least 12 hr. Remove paraffin with xylene. Treat with abs. alcohol, then with 1.0% celloidin in a mixture of alcohol and ether 1:1. Allow to partly dry. Place in 90% alcohol and stain as usual.—*S. H. Hutner.*

LEACH, E. H. **Fat staining with Sudan black B.** *J. Path. & Bact.*, **47**, 635–7. 1938.

Sudan black B (I. G. Farbenindustrie) in 50% diacetin (glycerol diacetate) stains nuclei red; lipid substances, including myelin, stain blue-black. Tissue should be fixed in 5% formalin in 0.9% saline for 24 hr., or in Zweibaum's fluid, made by mixing equal parts A and B. Sol. A: 3% $K_2Cr_2O_7$, 6 cc.; 2% CrO_3, 3 cc.; dist. water, 5 cc. Sol. B: 2% OsO_4. After fixation wash 24 hr. in running water. Embed in gelatin and cut frozen sections. Transfer 5-10μ sections from dist. water to 50% diacetin for 30 sec., preferably with agitation. Stain 15 min.–2 hr. in sat. soln. Sudan black B in 50% diacetin. The stain should be filtered before use. Staining may be shortened by warming in the paraffin oven without danger of precipitation of the dye. Transfer to 50% diacetin for 30 min., then float on clear water. Mount in Apathy's medium. The solution should be discarded when it starts to smell of acetic acid (caused by hydrolysis of the diacetin).—*S. H. Hutner.*

MONNE, L. **Über experimentell hervorgerufene strukturelle Veränderungen der Golgi-apparates und der Mitochondrien sowie über Bildung von Myelin-figuren in Spermatocyten und Spermatiden von Helix lutescens.** *Protoplasma*, **30**, 582–91. 1938.

In dist. water the mitochondria swell while the Golgi apparatus remains almost unchanged. The swelling of the mitochondria can be followed in a 0.4% hypotonic solution of NaCl containing pyronin. The stain colors only the periphery of the swollen mitochondria. In 0.73% isotonic NH_4Cl and in 0.7% isotonic NaCl containing NH_4OH the mitochondria remain unchanged while the Golgi apparatus undergoes marked structural changes suggesting liquefaction. The Golgi of the spermatids behave similarly.—*R. Chambers.*

MONNE, L. **Über Vitalfärbung des Golgi-apparates und der ergastoplasmatischen Strukturen in einigen Gastropoden-Zellen.** *Protoplasma*, **30**, 460–6. 1938.

A procedure is given for staining epithelial cells of the vas deferens, of the ducts of the receptaculum seminis and of the finger-like and salivary glands of *Helix*

lutescens, *H. pomatia* and *Tachea austriaca*. Fragments of the organs were placed for about an hour in 0.73% isotonic NH_4Cl, then for 15 min. in 0.8% isotonic NaCl and finally for 10–15 min. in 1.15% isotonic $NaHCO_3$ containing a fairly strong mixture of dahlia and chrysoidin in equal parts. The best results were obtained with the salivary gland cells of *Helix lutescens*. The cells are large and well suited for the investigation.—*R. Chambers.*

NIERO, G. **Sulla nuova tecnica di Bodian per le neurofibrille.** *Monit. Zool. Ital.*, **49**, No. 6, 149–53. 1938.

Method for material containing bony tissue: Fix in Bodian's formol acetic alcohol (formula 2) for 3 days or longer; wash in dist. water for several hours; decalcify completely in 4 to 5% nitric acid. Wash in dist. water for 48 hr. (keep slowly moving with Thoma's apparatus); dehydrate in the alcohol series and embed in paraffin or celloidin; impregnate the mounted sections according to Bodian. In sections of the spinal cord, the nerve cells with neurofibrillae, ganglion cells, and peripheral fibers are clearly demonstrated.

Method for impregnating dissociated intestinal wall: Fix the entire intestine using Bodian's formula 2, preferably for 36–48 hr.; dissociate and wash in dist. water rinsing for 4–5 hr.; immerse in a bath of 1% protargol (Protargol Bayer), containing 5 g. of metallic copper, for 5–6 hr. at 37° C. Reduce as indicated by Bodian. If desired, tone with gold. Mount in balsam or glycerin. With delicate impregnations, the sympathetic plexi as well as fibers and nerve cells can be demonstrated.—*A. B. Dawson.*

NITTIS, S. **The nature and the mechanism of the staining of the erythrocytic reticulum.** *Amer. J. Med. Sci.*, **196**, 177–9. 1938.

Reticulum in immature erythrocytes is a precipitate caused by the interaction between a basophilic substance in the cell and the vital dye. In counterstaining the reticulocytes with Wright's stain, the reticulum stain is replaced by methylene blue.—*J. A. Kennedy.*

RASMUSSEN, A. T. **The innervation of the hypophysis.** *Endocrinology*, **23**, 263, 1938.

A method is given for the staining of the hypophysis to bring out the nerve fibers more uniformly. The procedure is as follows: Fix small pieces in abs. alcohol containing 6 drops of NH_4OH per 100 ml., 2–3 days; wash in running tap water to remove ammonia, 12 hr.; treat with 3% nitric acid, 2 hr.; rinse in tap water, a few minutes to an hour depending on the size of the piece of tissue. Treat with several changes of 80% alcohol, a few hours; 80% alcohol+4 drops NH_4OH per 100 ml., over night; pure pyridine, 12 hr. Wash out pyridine with repeated changes of dist. water until no odor of pyridine is detectable. Treat with 2% $AgNO_3$ in the dark, 24 hr.; 0.75% $AgNO_3$ in the dark, 24 hr.; 2% $AgNO_3$ in the dark, 24 hr. Rinse quickly in dist. water; reduce in 4% pyrogallic acid, 24 hr.; dehydrate and clear (details not given); embed in medium paraffin so that sections can be cut at least 10 μ thick.—*L. Farber.*

ROBB-SMITH, A. H. T. **Reticulosis and reticulosarcoma: a histological classification.** *J. Path. & Bact.*, **47**, 457–80. 1938.

Lymph nodes removed at biopsy for histological examination should be fixed intact in 4% saline formalin for about 6 hr., then bisect the node and allow to fix thoroly. This avoids distortion due to capsular contraction if the node is bisected unfixed. Additional material may be fixed in other fluids.

Paraffin sections are stained with hematoxylin and azo-eosin, iron hematoxylin and Van Gieson stain (1%), and aq. toluidine blue to demonstrate basophil cytoplasmic granules.—*S. H. Hutner.*

ULPTS, R. G. E. **Morphologische Studien zur Physiologie der Erythrocyten und zur Blutplättchengenese.** *Folia Haematol.*, **60**, 205–21. 1938.

The technic permits the demonstration of the following constituents. 1. An outer zone containing hemoglobin. 2. An inner lyophilic hyaline body. 3. Fre-

quently observed paired granules. 4. A body which upon release into the blood stream simulates a blood platelet. 5. A "restbody". Guinea pigs furnish the most suitable erythrocytes.

Check blood by ear puncture and examination of 10 stained smears. Inject 0.5 ml. phenylhydrazin soln. (0.75 ml. phenylhydrazin base to 25 ml. dist. water). Make 10 additional blood smears at intervals of 24 hr. Fix a portion in methanol and stain 30 min. in Giemsa. Fix the other portion in Dominici's fixative (aq. sat. soln. $HgCl_2$ and tinct. iodine, 20:2; shake well, filter, and add 2 parts of formalin). The presence of an erythroblast in a smear indicates the proper time for proceeding. Injection may be repeated in 72 hr. if no erythroblasts appear. *Procedure:* Anesthetize the animal. Remove the sternum high enough to expose the trachea and heart. The assistant cuts all the cervical vessels in one operation, simultaneously the operator plunges the 5 ml. syringe full of Dominici's into the left ventricle, and empties the contents into a porcelain dish with 1000 ml. of normal saline and transfers to a tall vessel to permit settling. The supernatant fluid is drawn off and replaced with dist. water. Invert the vessel twice to remove excess fixative. After settling out, draw off again. From the brownish material prepare smears. The air dried preparations take a Giemsa or a silver impregnation stain. Prepare smears of the rapidly fixed erythrocytes adhering to the endocardium by touching coverslip to the open heart. Alkaline Giemsa for 24 hr. and silver impregnation by Gordon's method gave best results.—*J. M. Thuringer.*

PLANT MICROTECHNIC

BELL, H. P. and FACY, V. **Microtechnic for winter buds.** *Canadian J. Res.*, Sec. C. **15,** 129–34. 1937.

The authors give rather detailed procedures for preparing winter flower bud material for microscopic examination, including directions for embedding. Alcoholic stains were used to help in fixing sections onto the slide. Safranin in 50% alcohol was used for nuclei and acid fuchsin in 70% alcohol for a general stain.—*G. I. Wallace.*

HUTCHINS, H. L. and LUTMAN, B. F. **Spine development on the spores of Ustilago zeae.** *Phytopathology,* **28,** 859–60. 1938.

The growth of a spiny wall from a gelatinous matrix during development of the smut spores has been a controversial point for some time. The following technic, used for staining the sections of corn bolls infected with the mycelium of *Ustilago zeae*, differs markedly from the Hutchinson stain (*Phytopathology*, **26,** 293–4, 1936) from which it was derived.

Fix material in Allen-Bouin mixture, cut and transfer sections to water. Stain 24 hr. in sat. orseillin soln. (1 g. of dye in 20 cc. 3% acetic acid). Wash quickly with 50% alcohol to remove excess stain. Stain 24 hr. in sat. anilin blue soln. (1 g. of dye in 100 cc. 3% acetic acid). Dehydrate with abs. alcohol, xylene, and mount in balsam. A permanent differential stain is obtained in which the exospore stains red, and later the red spines protrude into the blue layer of the primitive wall.—*J. A. de Tomasi.*

MÜNCHBERG, P. **Ein auch für den Mikroskopier wertvolles Verfahren zur Konservierung von grünen Pflanzen und deren Teile.** *Mikrokosmos,* **32,** 14–5. 1938.

The method of v. Pillich and Winkler, developed primarily for the preservation of botanical materials in their natural colors, was found suitable for histological investigation after rinsing the material in water and dehydrating in alcohol.

Place material in large vessel containing sufficient 0.5% aq. zinc sulfocarbolate solution to cover specimens and exhaust the air for 20–30 min. Tap water may be used because it does not react with Zn ions to form precipitate. The material is now transferred to a tall cylindrical vessel half filled with a solution containing 1% zinc sulfocarbolate and 2% sodium benzoate. The filling of the vessel is now completed with a solution of 2% Na_2SO_3 and sodium benzoate (meaning 2% of each solution?). The fine precipitate formed is siphoned off in a week and the material transferred to 10% HCl in which the adhering precipitate is removed. After washing the material in water it is returned to the filtered solution in the

tall vessel. Zinc sulfocarbolate was selected because it was found to form a leuco-compound with chlorophil, while the sodium benzoate merely acts as a preservative and bactericide. The Na_2SO_3 neutralizes the zinc sulfocarbolate (after hydrolyzing).—*J. M. Thuringer.*

WOLF, F. T. **Cytological observations on gametogenesis and fertilization in Achlya flagellata.** *Mycologia*, **30**, 456–67. 1938.

Cultures of this fungus were maintained on boiled hemp seed in dist. water. Sexual organs were produced in abundance. For fixation, best results were obtained with mixtures of 0.3% chromic acid and 0.7% glacial acetic acid, diluted with dist. water until plasmolysis was reduced to a minimum. Embed in paraffin, cut sections 5 μ or 10 μ in thickness, remove paraffin in xylene, and pass slides down graded series of alcohols to water. Treat slides as follows: K-KI 20 hr. (no conc. given), 5 min.; wash in water a few seconds; crystal violet sol. (no conc. given), 30 sec. to 5 min.; wash in water; K-KI soln. (no conc. given), a few seconds; wash in water; flood slide with 95% alcohol and drain; flood slide with sat. picric acid in 95% alcohol and drain; flood slide with abs. alcohol; destain further in clove oil until desired color intensity is secured; clear in xylene; mount in balsam.—*Elbert C. Cole.*

WOODROFF, J. G. **Method for "fixing ice crystal patterns" in frozen products.** *Science*, **89**, 87. 1939.

It is difficult to obtain good paraffin sections of fruits and vegetables preserved by freezing. The tissues are very delicate and easily distorted. The author recommends the use of a freezing microtome, cutting at an average temperature of 18–20° F. Materials, microtome and supplies are chilled for 1 hr. before use. Where fine ice crystallization is produced, sections can be cut as thin as from 10–30 μ.

Kill and fix by floating sections in the following chilled formic-acetic-alcohol soln.: acetic acid, 5%; formalin, 10%; alcohol, 70%; water, 15%. This makes the material sufficiently stiff to prevent sticking together, to facilitate handling, and to assure satisfactory ice crystal patterns. The unmounted sections are best stained with eosin, orange G, and basic fuchsin for cell contents; with light green, basic fuchsin, and methylene blue for cell walls. Safranin with Delafield's hematoxylin gives best results for anatomical study of asparagus tips; and orange G, light green, or safranin-light-green for starch grains and cell walls of pears, beans and corn.—*J. A. de Tomasi.*

MICROÖRGANISMS

BISSET, K. A. **The structure of "rough" and "smooth" colonies.** *J. Path. & Bact.*, **47**, 223–9. 1938.

The morphology of bacterial colonies may be studied by means of impression preparations. Inoculate agar plates (blood agar is especially suitable) with a glass rod to avoid scratching the surface. When the colonies are 6–48 hr. old and the surface of the medium perfectly dry, press the agar surface against a cover slip. Fix by immersion in Bouin's fluid until the medium is bleached thruout (usually 2 hr.). Peel the medium away from the cover slip, leaving the colonies attached to the cover slip. Remove the fixative by soaking a few hours in water. The preparation is then stained as usual; dilute fuchsin is useful for those to be photographed.—*S. H. Hutner.*

CHAMBERS, S. O. and SCHOLTZ, J. R. **Clinical application of a stain for spirochaetes (Krajian).** *Arch. Dermat. & Syphilol*, **38**, 217–24. 1938.

The clinical use of the Krajian stain for spirochaetes has been found accurate for identifying *S. pallida* in cases of suspected primary syphilis. It has certain advantages which suggest a wider applicability than the dark-field procedure.—*J. A. Kennedy.*

COPE, E. J. and KASPER, J. A. **Cultural methods for the detection of typhoid carriers.** *Amer. J. Pub. Health*, **28**, 1065–8. 1938.

A comparison was made of the merits of a modified Wilson and Blair bismuth sulfite agar with Endo agar. The modified Wilson and Blair medium consists of: peptone, 1.0%; beef extract, 0.5%; dextrose, 0.5%; Na_2HPO_4, 0.1%; $FeSO_4$ 0.03%; $Bi_2(SO_3)_3$ indicator, 0.8%; brilliant green, 0.0025%; agar, 2.0%. A total of 2,273 examinations of feces and urine were made. In the finding of 452 positive cultures, the $Bi_2(SO_3)_3$ agar, when poured over 5 cc. of a heavy fecal emulsion, accounted for an increase of about 40% of the positive cultures. Endo agar accounted for the recovery of typhoid and paratyphoid bacilli from 23% of the number of positive specimens when $Bi_2(SO_3)_3$ failed. In view of the positive findings with Endo alone, Endo should not be discontinued, but should be supplemented by Bi agar for routine examination.—*M. W. Jennison.*

FITE, G. L. **The staining of acid-fast bacilli in paraffin sections.** *Amer. J. Path.*, **14**, 491–507. 1938.

Tissue should be fixed in alcohol, an alc. solution of formaldehyde, or 5% alc. $HgCl_2$. Mercury deposits are best removed by I_2 solution followed by alcohol and finally $Na_2S_2O_3$. After fixation in $K_2Cr_2O_7$, thoro washing of the tissue block and subsequent treatment of the section with $KMnO_4$ and oxalic acid is recommended. Acid-fast bacilli will not stain after prolonged fixation in either dichromate or formaldehyde solutions or passage thru decalcifying fluids.

Staining solution: New fuchsin (source of dye not given) 1 g., phenol crystals, 5 g., methyl alcohol, 10 cc., dist. water to make 100 cc. Dissolve the dye in the mixture of phenol and alcohol and add the water slowly. The staining solution keeps if protected from evaporation, but deteriorates rapidly in Coplin jars.

Staining time after alcohol fixation: At 20° C., 2–8 hr.; 37°, 1–4 hr.; 60°, 0.5–2 hr.; 90° (steaming), 5 min. After other fixation, 3 to 4 times longer except steaming, 5 min. Prolonged staining at room temperature is preferred. Differentiate with 1% HCl in 70–95% alcohol. Counterstain with methylene blue or hematoxylin if desired.—*H. A. Davenport.*

GUNSALUS, I. C. and STARK, C. N. **Formate ricinoleate and brilliant green bile broths to detect coliform organisms in milk.** *Amer. J. Pub. Health*, **28**, 832–4. 1938.

Formate ricinoleate broth was used to determine the presence of coliform organisms in 655 samples of pasteurized milk. Ninety-eight per cent of the positive presumptive tests confirmed; in the other 2% (3 samples) slow lactose fermenting or related organisms were found. In comparative tests on 221 samples of pasteurized milk, formate ricinoleate and brilliant green, 2% bile broth gave approximately the same number of positive presumptive tests. Brilliant green gave a slightly higher yield of positive presumptives, but some of these were shown to be due to anaerobic spore-formers.—*M. W. Jennison.*

HAGEMANN, P. K. H. **Fluoreszenzfärbung von Tuberkelbakterien mit Auramin.** *Münch. Med. Woch.*, **85**, 1066. 1938.

A procedure is given for the direct detection of tubercle bacilli in sputum, pus, feces, urine, etc., with the aid of the fluorescence microscope developed by the author (*Zentbl. Bakt., I Orig.* **140**, 184. 1937). The rather thin flame-fixed smears are stained as follows: 1:1000 aq. auramin "Bayer" containing 5% "phenolum liquefactum", 15 min.; wash well with tap water, decolorize with an acid alcohol solution (ethanol, 1000 cc.; conc. HCl, 4 cc.; NaCl, 4 g.), 3 min., renewing the alcoholic-HCl solution after 1½ min.; wash well with tap water. Examine the smear without cover glass with the fluorescence microscope, using 180× magnification, apochromatic dry objective and a 3× compensation ocular. For the absorption of the visible and red rays a 3.5 mm. thick "Uvet" lens and a 2% $CuSO_4$ solution are used.

The tubercle bacilli appear as golden yellow fluorescent rods, clearly differentiated from the violet fluorescent background. Eighty per cent agreement with cultural and animal inoculation methods is found. The sensitivity of the method may be increased by treating the material to be tested with antiformin or other enrichment agents.—*L. Farber.*

HOFFMANN, E. **Einfache Dauerfärbung von Hautpilzen und Mikroorganismen mit Azureosinglycerin.** *Klin. Woch.*, **17**, 1622–3. 1938.

A mixture of 2–3 parts of Azure-eosin (Giemsa) and 3–10 parts glycerin is prepared and a drop is placed on a slide with a few skin scrapings, cleared in 10–20% KOH before mounting. This stains the mycelia and spore masses of the fungi dark blue and the cornified epithelium rose. A piece of dead skin may be fastened to the slide with albumin fixative and stained in the same way. Tissues fixed in alcohol and sectioned, or air-dried smears fixed in 70% alcohol, may be stained over night in dilute solutions of Giemsa-glycerin, dehydrated in acetone, cleared in xylene, and mounted in cedar oil. The fungus then stains red, and the finer structures show very clearly. Other organisms, including pneumococcus, may be stained; other tissues also stain satisfactorily.—*Virgene W. Kavanagh.*

ISADA, M. **Geisselfärbung aus alter Bakterienkultur.** *Zentbl. Bakt., I Abt. Orig.*, **142**, 480–3. 1938.

Flagella stains of cultures several days old were found superior to those of young cultures. Six anaerobes were incubated at 37°C. and held for 2–3 days at room or refrigerator temperature. Smears were made from the slants (not the condensation water) using a drop of physiological saline or dist. water on perfectly clean cover glasses which had been dried several hours in a drying oven. The formula used was that of Toda (*Nippon Izi Sinpo* **283**, 113, 1928). *Sol. A:* 20% aq. tannic acid, 5% aq. phenol, sat. aq. alum, 2:5:2. *Sol. B:* sat. alc. soln. of fuchsin or gentian violet, Victoria blue 4R, etc. Smears were stained 0.5–2.5 min. in a freshly mixed, refiltered mixture of Sol. A and Sol. B., 9:1.—*J. P. Sell.*

KRAJIAN, A. A. **A reliable method of staining Spirochaeta pallida in smears.** *Arch. Dermat. & Syphilol.*, **38**, 427–8. 1938.

A swab is moistened in alcohol and the lesion roughly rubbed until it bleeds. Several minutes after all the bleeding stops, a clear serous exudate will appear on the abraded surface; from this smears are prepared and dried in air. Each smear is flooded for 5 min. with a warm No. 1 solution [$UO_2(NO_3)_2$, 1 g.; formic acid (85%), 3 cc.; glycerin (C. P.), 5 cc.; acetone, 10 cc., and alcohol (95%), 10 cc.] washed in dist. water and treated with a weak solution of gum mastic (3 drops of sat. alcoholic solution of gum mastic mixed with 7 cc. of 95% alcohol) for 2 min. The gum is poured off and the breath blown over the surface of the smear, which is then rinsed in dist. water. The slide is set on a metal stand or tripod, flooded with a 1% aqueous solution of $AgNO_3$, heated over a burner until bubbles begin to form (not boiled) and kept at that temperature for 3 minutes. The silvering process is repeated once, the silver poured off without washing, a thin coat of developing solution applied and the slide left under electric light for 2 minutes, being warmed gently with a flame. The developing solution consists of hydroquinone, 0.31 g.; Na_2SO_3, 0.06 g.; 40% neutral, solution of formaldehyde, 2.5 cc.; pyridine, 2.5 cc.; sat. solution of gum mastic in 95% alcohol, 2.5 cc., and dist. water, 15.0 cc. The slide is then washed with dist. water, dried on filter paper and examined under an oil immersion lens. For permanent mounts the preparation may be dehydrated with abs. alcohol, blotted, cleared in xylene and mounted in dammar. Stained smears should be brown. No. 1 solution is stable and keeps for long periods. Ag solution should be prepared from a 10% stock solution each time. Dilute gum mastic should be prepared fresh. The developing solution keeps well for from 2–3 weeks in a light room, after which it deteriorates, the gum mastic separating and settling to the bottom of the container. When this occurs, a fresh supply should be prepared. It is important to use fresh developing solution; otherwise the organisms will fail to stain.—*J. A. Kennedy.*

McAULIFFE, H. D. and FARRELL, M. A. **Dye concentration in culture media employed for the analysis of Escherichia-Aerobacter members in milk.** *Amer. J. Pub. Health*, **28**, 1217–21. 1938.

Milk adsorbs a considerable portion of the dye from fuchsin lactose broth and from brilliant green lactose 2% bile broth, thereby lessening the selective action of these media. To obtain enough free dye so that they will be selective for coliform organisms, the content of basic fuchsin in fuchsin lactose broth should be increased from 1 part in 66,666 to 1 part in 9,500, and the content of brilliant

green in the bile medium should be increased from 1 part in 75,188 to 1 part in 30,000. The above dye concentrations in each case are, first, the concentration specified by Standard Methods of Water Analysis for water, and second, the authors' findings for the concentration in milk to give the equivalent of *free* dye specified for water. Studies with different milks are included.—*M. W. Jennison.*

RACICOT, H. N., SAVILLE, D. B. O. and CONNERS, I. L. **Bacterial wilt and rot of potatoes—some suggestions for its detection, verification and control.** *Amer. Potato J.*, **15**, 312–8. 1938.

In connection with a procedure for making smears from vines and tubers, the following modification of the Gram stain is suggested. It is reported to have been devised by G. B. Reed but not to have been published previously.

Prepare the following solutions: (1) 0.25% aq. crystal violet; (2) 1.25% aq. $NaHCO_3$; (3) 2% iodine in a mixture of 1 vol. M/1 NaOH to 9 vols. water (the water added after the iodine has been dissolved in the NaOH); (4) 4 vols. 95% ethyl alcohol in 1 vol. acetone; (5) 1 vol. sat. solution basic fuchsin in 95% alcohol to 9 vols. water.

Flood smear with equal parts of soln. 1 and 2 for about 10 sec., then drain off excess; flood with soln. 3 for about 10 sec. and wash in water; apply soln. 4 until no more color comes away (about 5–10 sec.) and wash in water; flood with soln. 5 for not over 2 or 3 sec., wash and dry.—*H. J. Conn.*

SANDIFORD, B. R. **A new contrast stain for gonococci and meningococci in smears.** *Brit. Med. J.*, **1**, 1155. 1938.

A Gram stain with Pappenheim's stain for counterstain is based on the methods of Fauth and of Scudder.

Stain: Crystal violet, 1 g.; 98% alcohol, 20 cc.; and 1% aq. ammonium oxalate, 30 cc. Leave on smear, 30 sec.; wash with triple strength Lugol's, 30 sec.; blot; decolorize with acetone, 3–4 sec.; wash; counterstain, 2 min.; flood with water (do not wash) and blot.

Counterstain: Malachite green (source not given), 0.05 g.; pyronin (Grübler), 0.15 g.; and dist. water, 100 cc. Cells and nuclei are stained bluish-green; Gram-positive organisms, purple-black; and Neisserieae, red.—*Sara A. Scudder.*

Standard Methods for the Examination of Dairy Products. *7th Ed. Amer. J. Pub. Health*, **28**, 1447–8. 1938.

The forthcoming edition of what was Standard Methods of Milk Analysis has been enlarged to include certain milk products. Bacteriological Methods has been entirely rewritten, with important changes in composition of the agar for the standard agar plate count technic. Methylene blue-thiocyanate tablets, having been standardized, are accepted as standard. Brilliant green lactose bile broth, sodium formate ricinoleate broth, violet red bile agar, and sodium desoxycholate agar are approved for use in detecting coliform bacteria in dairy products. Several changes in methods and new tentative methods for the detection of certain pathogens in milk are given. This new edition will be in print early in 1939.—*M. W. Jennison.*

STARKEY, R. L. **A study of spore formation and other morphological characteristics in Vibrio desulfuricans.** *Arch. Mikrob.*, **9**, 268–304. 1938.

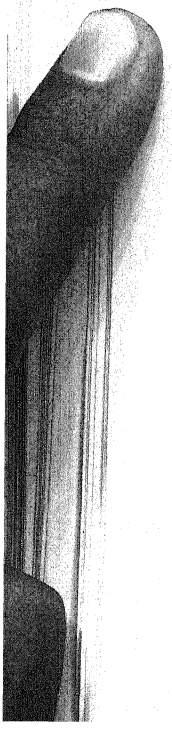
Dorner's method of staining spores was modified as follows: Flood the fixed smear with 5% CrO_3 and heat 30 sec. over boiling water. Wash, flood with carbol fuchsin and heat 10 min. over boiling water. Wash off excess stain, dry and spread over the smear a thin film of nigrosin. Spores are stained a deep red in colorless cells which show distinctly against a gray background. Early stages of spore formation can apparently be detected by this method.—*Merritt N. Pope.*

STEWART, H. M. **The staining of Trichomonas riedmüller, with Wright's blood stain.** *J. Parasitology*, **5**, 473–4. 1938.

Place material on a glass slide and fix with OsO_4 fumes by inverting it over a 5 ml. beaker, in the bottom of which has been placed a piece of folded filter paper

(Whatman 5.5 cm., No. 5). Immediately before use, place on the filter paper 1 or 2 drops of 2% OsO_4 soln. in 1% CrO_3 soln. Expose the Trichomonas to the osmic acid fumes until microscopic examination shows no motility among the organisms (10–30 sec. is sufficient). Air the smear . Cover smear with 5 drops of Wright's stain. Add 15 drops of Sørensen's buffered soln. pH 7.0–7.6. The solution is mixed by tilting the slide gently 2 or 3 times. Allow the diluted stain to stand 2 min. It is then "floated off". Wash the slide thoroly with the Sørensen's soln. previously used as the stain diluent. Air dry the preparation.

The anterior flagella and those bordering the undulating membrane stain pink; cytoplasm, blue; posterior part of the axostyle, pink; that portion extending thru the cytoplasm, blue; blepharoplasts, chromatin ring around the posterior portion of the axostyle, endoaxostylar granules and nucleus, dark purple. The cystostome appears as a clear area at the anterior extremity of the body of the organism.—*Elizabeth Bachelis.*



STAIN TECHNOLOGY

VOLUME 14 JULY, 1939 NUMBER 3

PROGRESS IN THE STANDARDIZATION OF STAINS

STANDARDIZATION OF STAINING SOLUTIONS

In the last issue of this journal, attention was called to the fact that the forthcoming edition of the National Formulary is to contain formulae of staining solutions which have been standardized in coöperation with the Stain Commission. Still another step is shortly to be taken in the direction of standardizing staining solutions which will be of interest to those users of stains who find it most convenient to buy their solutions already prepared.

Two recent notes[1] in the Journal of the American Medical Association have contained preliminary announcements of this plan. The plan is not yet quite ready to put into operation, altho it is hoped that it will be before the end of the current year. When it does go into effect, it will work out something as follows:

A selection is being made at present of a number of reliable concerns thruout the country which prepare and sell staining solutions. When this list is completed, it is hoped to have some such concern to coöperate with us in all of the large centers thruout the country. The names of concerns for this list are being selected upon the recommendation of members of the Stain Commission.

When the list is complete, it is planned to ask each concern to submit to us samples of certain staining solutions; at the start we will begin with only one or two of those (notably Wright stain) which are most frequently purchased in solution form. After testing such a solution, it is the plan to give the concern submitting it information as to whether it meets our tests and if not as to how it may be improved so as to do so. After any one of these concerns has thus demonstrated its ability to make up a satisfactory solution of any particular stain, we will furnish them labels to be used on this product stating essentially as follows:

> This certifies that this solution was made with dyes certified by the Commission on Standardization of Biological

[1]Conn, H. J. 1938. The work of the Commission on Standardization of Biological Stains. J. Am. Med. Assn. **111**, 1239–40.

————. 1939. Certification of staining solutions. *Id.*, **112**, 1281.

Stains and according to the directions of the National Formulary. It was prepared on.....

These labels will be sufficiently different in style from the regular certification labels so that they will not be confused with them. They will be sold to the dispensaries of the staining solutions at a small price per label.

As a check on the situation, it is planned to institute some kind of inspection system so that occasional samples of these solutions are purchased by Commission members on the open market and sent to the chairman's laboratory for testing. It is hoped in this way to make certain that the quality of such solutions is maintained.

It is also intended to publish the names, from time to time, of the concerns which are coöperating in this way, together with lists of staining solutions which have been put on this basis. This will enable users of such solutions to learn where they can purchase them.

Finances of the Stain Commission

A recent report in Science[2] has called attention to the fact that the Stain Commission is no longer to receive any financial assistance from the Chemical Foundation. This situation has been brought about because of the large decrease in the Foundation's income which has taken place upon the expiration of the patents that it has owned. It is in no sense to be regarded as due to loss of interest of the Chemical Foundation in the work of the Stain Commission. This is well evidenced by the following quotation from a letter just received from Mr. W. W. Buffum of the Foundation: "The Chemical Foundation is justly proud of the progress of the Commission on Standardization of Biological Stains. The total contributions by the Chemical Foundation from 1921 to date to your work have been $100,999.98, and we feel that your accomplishments during this period have more than justified this expenditure."

From the standpoint of continuing the work of the Stain Commission, it is unfortunate that the Chemical Foundation's appropriations must now be discontinued, but it is not so serious as it would have been a few years ago. For several years the size of these appropriations has been gradually reduced and the balance has been made up from the Commission's earned income. Had the Foundation been able to continue the partial support of this work, at a progressively reduced rate, for some five or six years longer, the probabilities are that by then the Commission would be entirely self-supporting. It

[2]Conn, H. J. *et al.*, 1939. Financial status of the Biological Stain Commission. Sci., 89, 367–8.

is accordingly possible at the present time to assure any other source from which funds are requested that they will be needed only to take care of a temporary shortage. Even without appropriations from any outside source, the deficit will not be great; a matter of $2,000 a year should balance the budget completely.

While it is much to be regretted that the support of the Chemical Foundation has to cease before the work of the Commission is entirely self-supporting, our feeling toward the Foundation is one of gratitude that it has been able to continue the support for so long—nearly twenty years, as a matter of fact. To appreciate the importance of this support, one should realize that when the standardization of stains was first undertaken, no one had any thoughts that it could be made self-supporting. The manufacture of stains was a very small branch of the dye industry which in itself was a "war baby", just getting on its feet. To have saddled the business of biological stain production with the expenses involved in standardization would have killed it at the start. Altho the National Research Council was very much interested in the project, they had no funds of their own available, and it was only because of the vision of the late Mr. F. P. Garvan and the generosity of the Chemical Foundation that the necessary financial support was forthcoming. It is not the least exaggeration to say that without this support, the work could never have been started. Accordingly, users of stains who feel that this standardization has been of value, owe a very considerable measure of gratitude to the Chemical Foundation for its assistance.

As we have every reason to believe that the work is going to prove self-supporting eventually, our chief hope at present is that some organization with a little money available will come to the rescue and tide us over the present emergency.—H. J. Conn.

THE PRESENT MEMBERS OF THE COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS

It has been several years since the last publication[1] of the list of members of the Stain Commission. In the meantime, there have been numerous changes, both in personnel and in addresses of some of the older members.

It has been even longer since any statement has appeared in these pages as to the way the Commission is organized; and as there are today many readers of STAIN TECHNOLOGY who did not see the early issues as they appeared, it seems well to repeat some of the information as to the make-up of this organization. The Commission was organized in 1922 by a Committee of the National Research Council. Its original membership consisted of about fifty collaborators who had been assisting this committee during the preceding year in its work on the standardization of biological stains. According to the constitution of the Stain Commission: "Other persons shall be eligible to membership who establish to the satisfaction of the Executive Committee that they are interested in the work of the organization, and are ready, willing and able to assist in the work of the organization, if and at such times as assistance in his or her special field may be requested of such member by the Executive Committee. Any such person may be admitted to membership upon his application to membership receiving the approval of the members of the Executive Committee. Continuance of membership shall be evidenced by the members signing and returning to the Chairman of the Executive Committee semi-annually a declaration in substantially the following form: 'I desire to renew and continue my membership on the Commission on Standardization of Biological Stains.' "

This latter provision of the constitution has been observed by sending out a card to each member twice a year bearing essentially the above statement; failure to return two of these cards in succession has been interpreted as equivalent to resignation.

The total number of members has been kept between 50 and 75 by adding new individuals occasionally to replace those who have dropped out. These new members have been added by invitation only.

The following are active members at the present time:

EXECUTIVE COMMITTEE

H. J. Conn, Agricultural Experiment Station, Geneva, N. Y.
E. V. Cowdry, School of Medicine, Washington Univ., St. Louis, Mo.

[1] STAIN TECHNOLOGY, 9, 73. 1934.

Louis Gershenfeld, Phil. College of Pharm. & Science, Philadelphia, Pa.
H. E. Jordan, Univ. of Virginia, University, Va.
S. I. Kornhauser, School of Medicine, Univ. of Louisville, Louisville, Ky.
F. B. Mallory, Boston City Hospital, Boston, Mass.
J. T. Scanlan, Bureau of Chemistry & Soils, U. S. Dept. of Agriculture, Washington, D. C.
W. D. Stovall, Univ. of Wisconsin, Madison, Wis.

Members at Large

C. E. Allen, Biology Bldg., Univ. of Wisconsin, Madison, Wis.
Ezra Allen, 450 E. 64th St., New York, N. Y.
Elizabeth Bachelis, 105 Elliot Place, Bronx, N. Y.
H. W. Beams, Dept. of Zoology, State Univ. of Iowa, Iowa City, Ia.
Wm. M. Bright, Municipal College, Louisville, Ky.
Victor Burke, Washington State College, Pullman, Wash.
E. Eleanor Carothers, Dept. of Zoology, State Univ. of Iowa, Iowa City, Ia.
C. J. Chamberlain, Dept. Botany, Univ. of Chicago, Chicago, Ill.
Clyde Chandler, N. Y. Botanical Garden, New York, N. Y.
G. H. Chapman, 604 Fifth Ave., New York, N. Y.
E. C. Cole, Williams College, Williamstown, Mass.
Francis W. Constable, St. Joseph's Hospital, Providence, R. I.
H. A. Davenport, 303 E. Chicago Ave., Chicago, Ill.
A. B. Dawson, Biological Laboratories, Harvard University, Cambridge, Mass.
J. A. deTomasi, 220 Plant Science Bldg., Cornell University, Ithaca, N. Y.
C. R. Fellers, Mass. State College, Amherst, Mass.
N. C. Foot, New York Hospital, 525 East 68th St., New York, N. Y.
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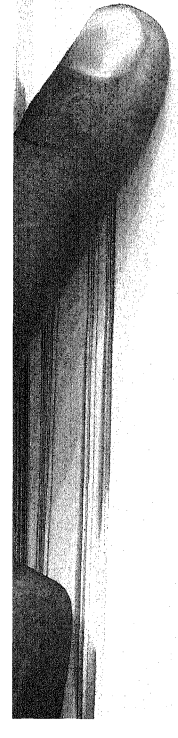
D. C. Hetherington, Duke Hospital, Durham, N. C.
E. G. Hood, Science Service, Dairy Research, Dept. of Agric., Ottawa, Canada.
Alfred E. A. Hudson, Waynesboro Hospital, Waynesboro, Va.
S. H. Hutner, 158 Knox St., Albany, N. Y.
D. A. Irwin, Banting Institute, 100 College St., Toronto, Canada.
M. W. Jennison, Biology Dept., Mass. Inst. Techn., Cambridge, Mass.
J. E. Kindred, Box 1341, University, Va.
Max Levine, Bact. Dept., Iowa State College, Ames, Ia.
Leon S. Lippincott, Box 231, Vicksburg, Miss.
H. Macy, University Farm, St. Paul, Minn.

W. L. Mallmann, Dept. of Bact., Mich. State College, East Lansing, Mich.
M. S. Marshall, Dept. of Bact., Univ. of Cal. Medical School, San Francisco, Cal.
C. E. McClung, Univ. of Pennsylvania, Philadelphia, Pa.
Roy L. Mobley, Box 1021, Baton Rouge, La.
J. T. Myers, Bendiner & Schlesinger Lab., 3rd Ave. & 10th St., New York, N. Y.
T. S. Painter, Univ. of Texas, Austin, Tex.
Alexander Petrunkevitch, Yale University, New Haven, Conn.
L. F. Randolph, Plant Science Bldg., Cornell Univ., Ithaca, N. Y.
P. W. Rohrbaugh, 549 Ocean View, Whittier, Cal.
Sara A. Scudder, City Hospital, Welfare Island, N. Y.
L. W. Sharp, Plant Science Bldg., Cornell University, Ithaca, N. Y.
Waldo Shumway, Univ. of Illinois, Urbana, Ill.
Karl A. Stiles, Coe College, Cedar Rapids, Ia.
F. W. Tanner, 362 Chemistry Bldg., Univ. of Illinois, Urbana, Ill.
Wm. R. Taylor, Dept. of Botany, Univ. of Michigan, Ann Arbor, Mich.
Alexander V. Tolstoouhov, 305 East 63rd St., New York, N. Y.
S. T. Walton, City Health Dept., Charlotte, N. C.
H. B. Ward, Univ. of Illinois, Urbana, Ill.
H. C. Waterman, 3218 Stephenson Place, N. W., Washington, D. C.



APPLICATION OF FINE GRAIN PROCESSING AND CONDENSER ILLUMINATION[1] ENLARGING TO PHOTOMICROGRAPHY

FRANK P. MCWHORTER, *Plant Pathologist, Oregon Experiment Station, Corvallis, Ore.*

ABSTRACT.—Photomicrographs involving great resolution are usually made by means of long initial projection. This involves the use of heavy, often cumbersome, apparatus designed to eliminate vibration. This paper evaluates the possibility of using an intermediate projection distance equal to or greater than 160 mm., which is considered the minimum efficient distance, and recording the initial negative image in very fine grain. Then by critical enlarging a positive image is obtained which closely approaches the resolution obtainable by the finest long projection equipment. The initial short projection permits elimination of vibration difficulties attending long exposures so that critical printing at magnifications above 2000 × will give consistently good results, provided fine grain technic is mastered by the operator.

Introduction: It is generally conceded that the limit of usable magnification for direct vision with compensating eyepieces is the numerical aperture of the objective multiplied by 1,000.[2] This goal is seldom attained satisfactorily even by experienced microscopists skilled in critical illumination methods because the human elements, eye strain, vision variations, and eye defects, restrict the final achievement. This article is intended to show how usable magnifications beyond the 1000 × aperture limit are obtainable with average eyepiece magnifications by combining recently developed fine grain processing with condenser enlarging. It is not asserted that such photographic magnification will increase resolution beyond the $\frac{\lambda}{2\,NA}$ law, but it is claimed that with a given set of lenses, these methods will (1) record details which cannot be seen with average vision and (2) enable any observer to notice details which he might not otherwise observe.

It is not claimed that the methods and ideas involved are new, nor that no other worker has ever combined fine grained processing with critical enlarging. Since the possibilities of the combination are not

[1]Published as Technical Paper No. 281 with the approval of the Director of the Oregon Agricultural Experimental Station. Contribution of the Department of Botany, and the Bureau of Plant Industry, U. S. D. A., coöperating.

[2]Belling, John. 1930. The use of the microscope. McGraw-Hill Pub. Co.

generally recognized, an analysis of the factors governing the results seems advisable.

Meaning of condenser illumination enlarging: The usual enlarger, including expensive "auto focus" models, is constructed with ground glass diffusion areas between the film holder and the light. Uniform illumination and partial diffusion for artistic effects are thus obtained. The light source is usually a frosted globe. The light scatters from the frosted surface in every possible angle, only a portion of it passing thru the film. In turn, the light transmitted to the lens is still diffusing. The lens must pick the rays moving in the right direction or turn those moving in the wrong direction into a cone which eventually reaches the enlarging paper. Most of the original light is wasted. If a properly adjusted condenser replaces the ground glass and an unfrosted concentrated filament lamp replaces the usual light source, we have the principles of critical illumination applied to photographic enlarging. The difference in performance is comparable to using a microscope with, versus without, a condenser; the optical principles are the same. The condenser in either case bends the light rays into a cone comparable to the numerical aperture of the lens system and eliminates unwanted diffused rays. A truly critical illumination enlarger is one in which the condenser system can be focused for the different positions which the projection lens assumes as different magnifications are obtained. For most purposes, however, one having a fixed condenser is very efficient. An excellent description of the optics involved was presented by H. C. Benedict in Camera Craft for June 1936.[3]

If a good negative portraying black lines close together is projected thru an ordinary enlarger, the lines will diffuse noticeably as the magnification increases beyond 2 ×, and will diffuse greatly beyond 3 ×. The better kinds of "Minicam" enlargers equipped with six element lenses but without condensers will project lines to 5 × with good effect. With critical illumination and microtessars, lines will project to 30 or more times—the emulsion permitting. In photomicrography in general, and certainly from the viewpoint of this article, we are dealing with line and dot projection; then even the best pictorial-purpose enlargers fail. Conversely, the more critical the enlarger, the more difficult it becomes to avoid graininess in the projected image.

The negative for enlarging: Photographs at high magnifications are usually obtained with special projection eyepieces and long bellows extensions necessitating elaborate mechanical devices to dampen vibration. The use of such equipment, altho time-consuming, is

[3]Benedict, H. C. 1936. A positive method for alternating the apparent exposure scale of a paper through the use of a condenser enlarger. Camera Craft, **23**, 275–284.

advisable for extreme resolution and magnification because objectives of high NA are cut with a theoretical projection point located at infinity. The image, however, when projected to a distance which permits very high initial magnification, tends to soften unless ideal illumination obtains. For this reason an image representing high initial magnification, unless the subject involves extreme contrast, requires a process film and a contrast developer for photographic recording. This concept is the basis for recommending the combination of the Wratten M plate and the developer D 19. (See footnote 2.)

The finest photomicrographs of minute difficult subjects are made with long initial image projection combined with tonal development as opposed to contrast development with D 19. This also permits secondary enlargement as described here. Dr. Max Poser's magnificent pictures of diatoms printed as glass transparencies at magnifications of 10,000 and more involve just such technic. Such work can be done only with exceptional equipment and by exceptional workers. The writer believes that anyone familiar with critical illumination principles can make good high magnification images more easily if relatively short initial projection distances are used in conjunction with fine grain processing. For routine work, a "ride the microscope" type of camera is advised. This type of camera accommodates plates up to the 9 × 12 centimeter size. If the microscope is lighted as in critical illumination and an eyepiece is used which is suitable for either looking thru or projection, then the good illumination which obtains for direct vision is also fine for projection, and very sharp images are present at 160 mm. from the microscope. Vibration difficulties, moreover, are largely eliminated.[4] The 160 mm. projection of the image represents an intermediate distance permitting the projected field to cover a 3¼ × 4¼ plate. It is far more usable than the "postage stamp size" initial images obtained by inverting a "candid" camera a short distance above a microscope. The making of a negative is a matter of moments—not hours. If the image is picked up at 160 mm. as with a Makam camera, the maintenance of contrast is less difficult and developers permitting gradation and tone rendering may be used; these developers may be chosen to eliminate graininess. The procedures herein recommended are based on the fact that the resolving power of a photographic emulsion is inversely proportionate to its graininess.

Tests of different developers: In order to obtain high magnification from relatively short initial projection, the negative must have fine grain and normal contrast. Unfortunately, the degree of gamma

[4]McWhorter, F. P. 1927. A simple and inexpensive method of making photomicrographs. The Camera, Dec. 1927, 381–385.

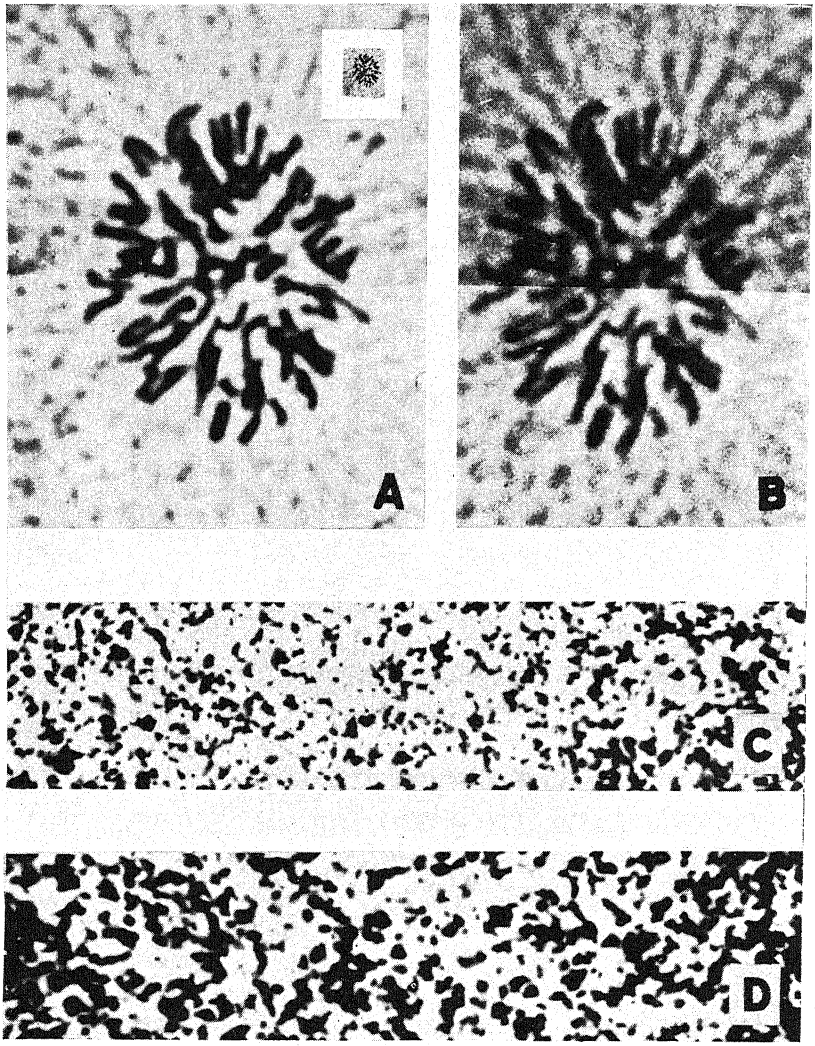


FIG. 1. *A* and *B* are photomicrographs of a transaxial section of a metaphase within a whitefish egg, projected 10 × from 0.65 NA, 200 × negatives. The final magnification is 2,000 ×, the secondary increase being denoted by comparison with the inserted direct print accompanying *A*. The upper part of each is printed darker to show the fibrilla structure of the cytoplasm which is just beyond the resolution of the lens but may be surmised from the record obtained thru the fine grain process shown in *A*. *A* is from a process panchromatic negative processed with Champlin 15 developer. *B* represents a combination of film and developer which is unsatisfactory for secondary resolution. Figs. *C* and *D* compare the emulsions composing the image of the chromosome shown at the bottom of Fig. *A*, but processed in Ultra Fine Grain, with the image of the same chromosome on a film processed in D 72, these being the coarsest and finest grains, respectively, encountered in these tests. The magnification of *C* and *D* is 1000 × and was obtained by photographing the image on the original negatives with the 0.65 NA lens and fine grain processing. Note the large difference in the size of the silver particles.

which obtains for any developer by varying the time and temperature is inversely proportionate to the fineness of grain. The emulsion of process panchromatic films and Wratten M plates is so fine *per se* that contrastive development in such formulae as D 19 and D 72 permits contact printing and mild enlarging with diffusion (ground glass) enlargers without any objectionable graininess. Fine grain developers make the grain of the finished image on fine grain films proportionately finer. Eastman process panchromatic film was used in these tests as a standard in determining the feasibility of different fine grain formulae for photomicrographic purposes.

TABLE 1. TABLE COMPILED FROM STUDY OF SEVERAL NEGATIVES TO SHOW THE UTILITY OF REPRESENTATIVE FINE GRAIN DEVELOPERS FOR PHOTOMICROGRAPHIC PURPOSES*

Developer	Exposure time seconds	Developing time minutes	Temperature of developer	Contrast and tone rating	Practical limit of critical enlargement for 8 mm. lens negative
D 72	2	7	70	Good	2–4 ×
D 76	7	7	70	Excellent	4–5 ×
Edwal 20	10	21	65	Good	4–5 ×
Infinol	9	15	68	Good	4–5 ×
Sease III	10	23	65	Good	4–6 ×
Champlin 15	10	21	70	Excellent	8–12 ×
Eastman Ultra Fine Grain	10	20	65	Poor	10–15 ×
Eastman Ultra Fine Grain	7	40	65	Good	8–10 ×

*These results are based on comparable photographs of a mitotic figure in whitefish eggs. D 72, which is less contrasty than D 19, is included as a standard of comparison. The data for the practical limit of enlarging are based on a study of a large number of negatives. This limit is a function of the initial contrast of the subject and of the resolving power of the emulsion as effected by different developers.

Fine grain principles are easily applied to low magnifications. For high magnifications, it was necessary to discover a developer that might be classed as normally fine grain and normally high contrast. Table 1 shows the result of comparative tests using as photographic subject a transaxial section of the metaphase of a mitotic figure in the egg of a whitefish. To permit further comparison by other workers, a typical slide sold by the General Biological Supply House, Chicago, Ill., under the number E 1378 was used.

The data in Table 1 are based on what may be termed normal development time and temperature for each of the developers.[5]

[5]With the exception of the last figures for Eastman Ultra Fine Grain.

Where the subject has great contrast because of initial constitution or contrasty staining or selective light filtering, any of these developers, including Sease III and Eastman Fine Grain, can be used at these normal times and temperatures. If the final (enlargement) magnification is to be very high, one of those listed as being very fine grain must be used. Fig. 4 shows how negatives developed with representative developers behave when projected to 10 × thru a condenser enlarger.

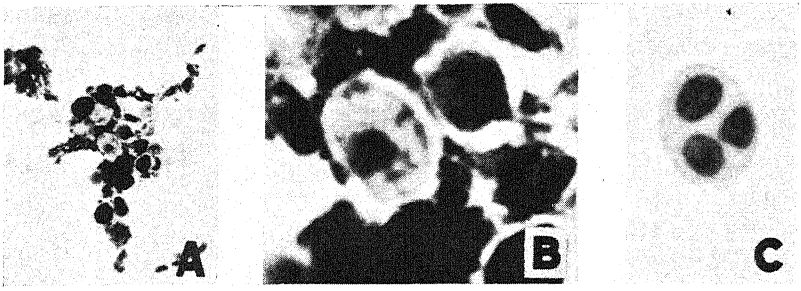


FIG. 2. *A* and *B* are yeast cells stained in Giemsa stain to show the very small nucleus. *A* is a direct print from 1.40 NA, process panchromatic Champlin processed negative at a projection of 160 mm. *B* is a print from *A* enlarged to 3,000 ×, the magnification scale being 3 mm. = 1 μ. *C* is from a methylene blue preparation photographed with a 3 mm. 1.25 NA lens to show spores and printed to show the area of the spore on the right which represents nuclear content. Emulsion-resolution permits prints which can be accurately measured for comparison of cell parts in difficult subjects such as yeast.

Choice of fine grain developer: Different emulsions respond in different degrees to various fine grain formulae. If vibration of the mechanism or movement within the microscope field forces one to use a fast emulsion, a series of tests is necessary to discover the best formula for the particular film involved. Eastman Panatomic film, for example, altho truly fine grain for macroscopical subjects, cannot be used satisfactorily even with the Champlin formula No. 15, if the film is to be subjected to condenser enlarging at over five times. This film can be so used if the Eastman Ultra Fine Grain developer is employed at a gamma rating of approximately 0.80. The new developer Infinol may prove useful for fast films. This subject will require investigation by each worker to meet his special requirements, especially since new fine grain formulae are continually being developed.

As stated above, any of the fine grain developers mentioned is satisfactory for process panchromatic film where secondary enlargement does not exceed 5 × and initial magnification does not exceed 300

diameters.[6] It is claimed by originators and makers of fine grain formulae that enlargements up to 30 × or even 60 × are possible. Such statements apply to pictorial enlarging only. Critical illumination enlargements show the outlines of individual crystals even in process films when any of these developers is used if the projection exceeds 15 ×. Graininess which would be unnoticed in macroscopic subjects, moreover, becomes very objectionable in photomicrographic records. For convenience, it is very desirable that developing times

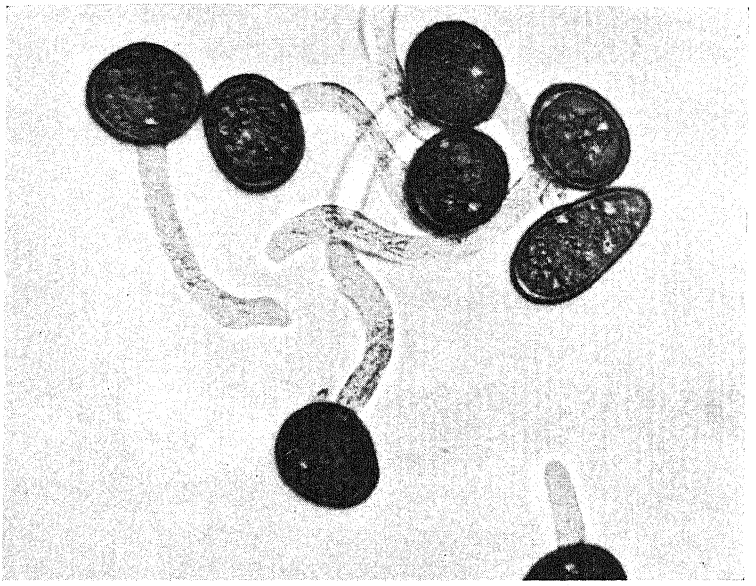


FIG. 3. Urediospores of *Puccinia convolvuli* germinating. The negative was made with an 0.65 lens, and processed in Champlin to show the textural rendering of the hyphae. The original magnification was 200 ×. The print represents a 5 × enlargement from the negative reduced one-half for making this illustration. Final magnification 500 ×.

should not exceed 25 minutes. In our tests, the Champlin No. 15 formula produced the highest contrast and proportionately finest grain[7] in the shortest time. The Eastman Ultra Fine Grain when

[6]The practical limit of secondary enlargement is also affected by the initial magnification which is proportionate to the magnification of the objective and in turn limited by the NA of the objective. The higher the initial magnification the more difficult it is to obtain negatives which will stand great secondary enlargement. For average subjects negatives made with oil immersion lenses should be developed to a fairly high contrast if secondary enlargement is to be attempted.

[7]Champlin, H. 1937. Champlin on fine grain. Camera Craft Pub. Co.

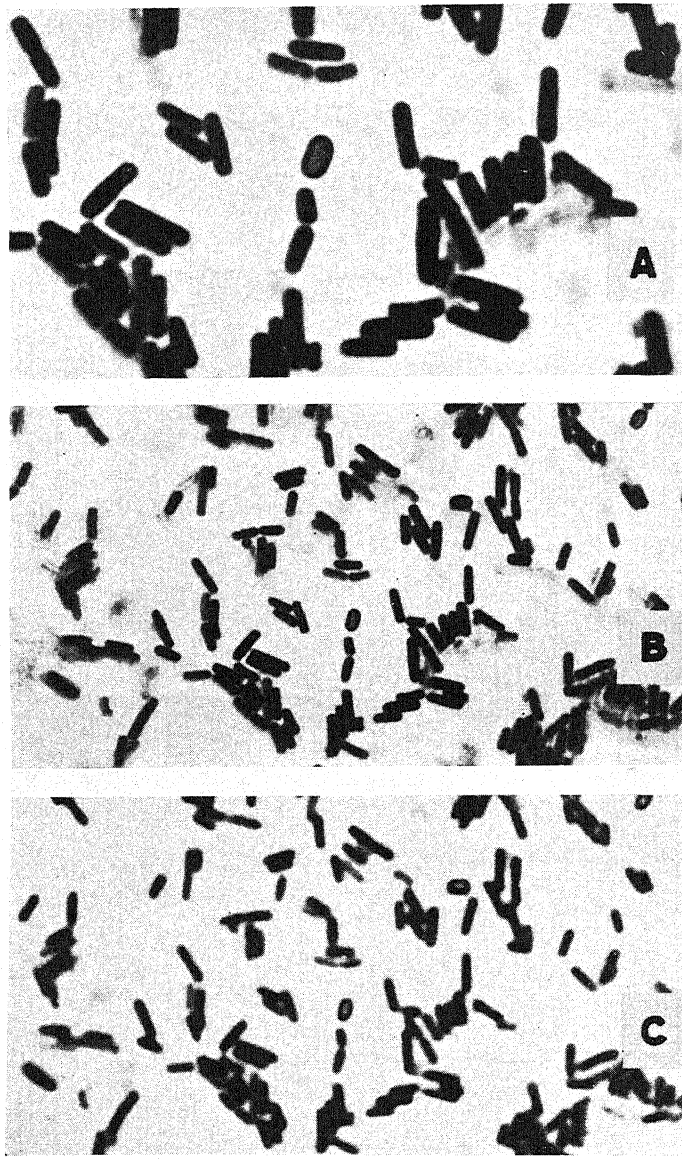


FIG. 4. Photomicrographs of spore bearing *Bacillus prausnitzii* stained with basic fuchsin. *A* and *B* are from a negative made with a 3 mm. 1.40 NA lens at an initial projection of 360 mm. developed to high contrast in D 72. *B* represents a non-critical enlargement of 2½ × bringing the magnification up to 2,000 ×. *A* is a critical enlargement from the same negative increasing the magnification to 5,000 ×. *C* is made with an 8 mm. 0.65 NA lens at an initial projection of 160 mm. and critically enlarged ten times to 2,000 ×. The negative was processed in Champlin 15. Note that *C* compares favorably with *B*, but that *A* is noticeably superior to either of the others. The spore plainly shown in *C* may be considered to be theoretically beyond the resolution of the 0.65 N. A., 8 mm. "dry" lens which was used in making the negative.

used at the top of its gamma curve (30 minutes at 65° F.)[8] develops less contrast than Champlin No. 15 at 20 minutes and then evidences noticeable graininess. Where very high secondary magnification is desired either of these developers may be used to good advantage but with the following difference in choice: The Champlin formula seems best for general subjects, as for example, bacteria, chromosomes, or general views of stained sections; the Ultra Fine Grain developer is excellent for determining the shape of particles which are on the border line of visibility and is very usable at low magnifications where excess contrast should be avoided.

The effect on the emulsion speed is another important point to consider in choosing a fine grain developer. The Champlin formula in our tests is only one-third slower than D 76; that is, the exposure for comparable results need be increased only one-third. Infinol proves faster than Champlin No. 15 but the coarseness of the grain in process panchromatic emulsion makes it inferior for critical work. The other developers are noticeably slower.

Notes on the Champlin formula: For average subjects when using process panchromatic films, we have found the Champlin No. 15 formula to be the most satisfactory for obtaining very fine grain. It does have several objectionable features and unless one is careful in preparing and using the developer very unsatisfactory results may obtain. The detailed discussion in Champlin's book and in his supplementary article[9] should be digested before one attempts to experiment with the formula. For photomicrographic application the following suggestions may be helpful:

(1) The developer must be made up in distilled water. The temperatures suggested by the originator should be followed very closely in preparing the developer.

(2) The exposure for process panchromatic film should be such that the negative assumes good density and strong contrast when developed for 20 minutes at 71° ± 1° in *fresh* developer.

(3) For tray development one may either use a small amount of the developer once or twice and then throw it away or else use a large volume several times and note the lag in rate of development, which accompanies its use. The lag in subsequent development is one of the chief objections to the developer.

(4) One must use extreme care to prevent this developer from touching clothes; it stains badly.

If these precautions are followed, fine grain developers, such as this one, open a new field in photomicrographic work.

[8]Vittum, P. W. and Crabtree, J. T. 1935. A new fine grain developer. Camera Craft, 22, 587–597.

[9]Champlin, H. 1937. Concerning Champlin 15. Camera Craft, 44, 426–529.

Other advantages of fine grain: Beginners in photomicrographic work tend to make low power negatives entirely too contrasty. When half-tones are made from such pictures they often fail to show the points involved. If fine grain developing is used, the negative is certain to maintain better tonal values; these can be emphasized as desired in printing.

Fine grain developers are very useful in photographing hyalin objects such as fungus hyphae mounted in water. This use we had not anticipated. Formerly we used D 19 thinking that the highest possible initial contrast would be necessary. The explanation seems to be that the fine grain records the texture of the subject which corresponds to the eye interpretation. A white mycelial thread examined in clear water presents visually a textural difference rather than a color difference. The use of a contrasty developer attempts to exhibit a contrast which does not exist. The fine grain image, being a record of texture, is far more lifelike.

Applications and illustrations: The negatives for the illustrations in this article, except Fig. 2, were made with an 8 mm., 0.65 NA lens. The initial magnification of such a lens is 20 × and the theoretical usable magnification is 650 times. The eyepiece limit would be 33 ×. The accompanying illustrations made with this lens are at magnifications of 1000 to 2000 ×. The result equates using a 50 × eyepiece or even a 100 × eyepiece. Every picture, except where indicated, is made by using a Makam camera, process panchromatic film, Champlin No. 15 developer and a condenser enlarger. As stated in the introduction, no claim is made that such a combination increases theoretical resolution but the following fact may be observed in Fig. 4, C, showing spores of bacteria photographed with the 8 mm. lens at a final magnification of 2,000 ×. This photograph represents an amazing performance for such a lens since these spores are less than 0.5 μ across and the theoretical limit of resolution for the lens with white light is 0.85 μ.[10] Few bacteriologists would dream of using an 8 mm. lens to observe spores in bacteria. These same methods of processing combined with ultraviolet light, quartz objectives, suitable eyepieces, and ideal initial projection distances should permit usable magnifications of 5,000 × to 15,000 ×. Theoretical resolution will not be increased, but usable resolution may, because fine grain processing enables the resolving power of the emulsion to keep pace with the resolving power of the lens system. For average equipment, however, fine grain processing will prove more readily adaptable to intermediate objectives than to the oil immersion series with the exception of the new 4 mm. oil immersion 1.00 NA lens recently issued by an American optical company.

[10]Seifriz, William. 1937. Methods of research on the physical properties of protoplasm. Plant Physiology, 12, 99–116.

IMPROVEMENTS IN THE PERMANENT ROOT TIP SQUASH TECHNIC

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Corn Syrup Mounts: In a previous paper[1] the author recommended a mixture of acetic acid and corn syrup as a simple and convenient mounting medium for root tip squash preparations. In work carried on since the publication of that paper it has been found with certain samples of corn syrup that the stain loses its brilliance and, in some cases, fades badly. This occurs only with the acetic acid corn syrup mixture and not with a water corn syrup mixture.

The conclusions of the previous paper were based on slides made in October, 1936. These slides are still in perfect condition, and no difference can be detected between those mounted in acetic acid corn syrup and corn syrup diluted with water. A series of very valuable slides, however, made in October, 1938, and mounted in the acetic acid corn syrup mixture, became worthless after two months. In this case a different lot of corn syrup was used; and the same thing has occurred with other samples.

The acetic acid corn syrup mixture was used because the acetic acid hastens penetration of the corn syrup into the cytoplasm and produces at the beginning clearer and sharper mounts, which, for immediate observation, are much better than water syrup mounts. After standing for a day or two, however, there is no observable difference between the two. Therefore, considering the variability of commercial corn syrups and their action with acetic acid, it is advisable, in order to ensure satisfactory results with this mounting medium, to use syrup diluted with water only, and the proportion of 1 part of corn syrup to 1 part of water is recommended.

Dioxan-balsam Mounts: One of the advantages of the corn syrup method is that separation and spreading of the cells is easily carried out in this medium, but this is not always the case with dioxan-balsam, as some root tips resist squashing after dehydration. This is due to the pectic acid, on dehydration, forming a hard compound which cements the cells together. Thus when one attempts to divide the root tip into longitudinal sections by means of needles, it breaks up into irregular pieces which are difficult to squash.

In order to overcome this difficulty it is necessary to remove the

[1]Hillary, B. B. 1938. Permanent preparations from rapid cytological technics. Stain Techn., **13**, 161–7.

pectic substance of the middle lamella. Cormack[2] has shown that the middle lamella below the zone of root hair formation consists mainly of pectic acid. This can be removed by treatment with bases such as NH_4OH and NaOH which convert the pectic acid into a soluble pectate. It was found necessary to apply this treatment before hydrolysis, otherwise a poor stain resulted. Apparently the aldehyde or color producing groups which are liberated by the acid hydrolysis undergo chemical degradation in the presence of a base, thus interfering with the Feulgen reaction.

Various concentrations of NaOH and NH_4OH were tried at different temperatures for varying periods of time. NaOH is not only unreliable but is likely to damage the cell structure seriously and to interfere with the staining. NH_4OH, on the other hand, brings about a clear-cut separation of the cells, without damaging them, and any left in the tissue is easier to remove as the NH_3 volatilizes. Of the different combinations of time, temperature and concentration used, 4% NH_4OH at 60° C. for 15 minutes, then 30 minutes washing, as pretreatment before the application of the Feulgen technic, produced the best preparations.

If it is desired to study the variations in the shape of nuclei which are to be found in the region of elongation, a different means of removing the pectic substance is necessary, since the middle lamella at this region consists of calcium pectate. This can be removed by ammonium oxalate which converts the calcium pectate into calcium oxalate and ammonium pectate, both of which are soluble and easily washed out. Treatment with 4% ammonium oxalate for 40 minutes at 60° C. before hydrolysis will bring about a separation of the cells and will not interfere with the stain. This treatment will also bring about a separation of the cells at the zone of active division, but it is not as rapid nor does it produce as good results as NH_4OH.

The procedure recommended for making permanent dioxan-balsam preparations of root tips is as follows:

1. Fix 30–60 min., preferably in a Navashin type fixative.
2. Wash 30 min. or longer in running water.
3. Treat 15 min. in 4% (by volume) NH_4OH at 60° C. in a corked vial (to prevent evaporation of the ammonia).
4. Wash 30 min. in running water to remove soluble pectate.
5. Hydrolyse 8 min. in *N* HCl at 60° C.
6. Stain 20 min. or longer in fuchsin-sulfurous-acid.
7. Bleach in 3 changes of SO_2-water for at least 10 min. per change.
8. Dehydrate in 3 changes of dioxan of 3 min. each.
9. Place a root tip in a drop of dioxan-balsam on a slide, separate into fine longitudinal strands by means of sharp needles.

[2]Cormack, R. G. H. 1935. Investigations on the development of root hairs. New Phytol., 34, 30–54.

10. Put on a cover slip, apply pressure to spread the cells and complete their separation by gentle tapping of the cover slip with a needle holder.

The addition of the NH_4OH step to the procedure easily allows one to make from any type of root tip, clear, sharp, and brilliantly stained preparations which show plates of cells one cell thick, filaments of cells, and individual cells.

DIOXAN DEHYDRATION FOR PARAFFIN EMBEDDED FUCUS SLIDES

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Good *Fucus* slides are difficult to prepare using the paraffin method, and those available commercially are often poor. Some supply houses ask a small premium price for celloidin prepared slides of *Fucus*. Excellent paraffin preparations can be made, however, using the slow dioxan method[1] and staining with fast green F. C. F. and safranin. (See figure.)

Because of the toxic properties of dioxan, certain precautions are advisable. In the writer's laboratory a large series of bottles is never

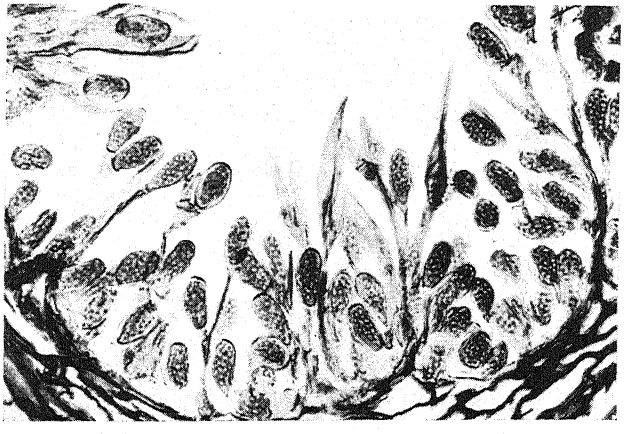

FIG. 1. Photomicrograph showing detail of *Fucus* conceptacle prepared using method herein described.

handled at a time and the containers are kept tightly closed as much of the time as possible. This prevents the fumes from escaping into the room for a considerable length of time; and if ordinary precautions for ventilation are taken when containers are opened, it is felt that there is no danger from the use of this reagent.

Fresh plant material was processed in a chromic-acetic-formalin fluid. (Solution A: 1% chromic acid 87 cc., glacial acetic acid 13 cc.; Solution B: comm. formaldehyde 20 cc., water 80 cc.; an equal quantity of each solution was used in the processing fluid.) After

[1]Ralph, Paul. 1938. A comparative study of some dehydrating and clearing agents. Stain Techn. **13**, 9–15.

24 hours in the killing and fixing fluid, the following dehydrating schedule was followed: Dioxan 33% and water 67% for 2 hours; dioxan 67% and water 33% for 2 hours; two changes of 100% dioxan, 1 hour each.

Paraffin infiltration followed McWhorter and Weier's[2] procedure except as to the time the materials were left in the solutions. Following the second change of anhydrous 100% dioxan the pieces of *Fucus* were placed in infiltration solution 1 (50 cc. dioxan, 5 cc. xylol, and 20 cc. of melted paraffin). The paraffin was allowed to "cake" and the liquid beneath the solidified paraffin was allowed to remain on the tissues at room temperature for 4 hours. The pieces of *Fucus* were transferred from solution 1 to infiltration solution 2 (50 cc. dioxan, 10 cc. xylol, 50 cc. melted paraffin). The material was then placed in oven (50° to 52° C.) for 2 hours. The next two changes were to soft paraffin for 2 hours each followed by embedding in paraffin in the usual way.

Sections cut 10μ thick were fixed to the slide and run thru the following staining schedule: 100% xylol; 100% xylol; 95% alcohol; 60% alcohol; water; aqueous safranin[3] (about 0.5%) for ten minutes; water; 50% alcohol; 95% alcohol; fast green F. C. F.[4] (about 0.25% in 66 cc. of 95% alcohol plus 33 cc. of anhydrous acetone). The slides were left in the fast green long enough to differentiate the reproductive organs. Following the fast green, the slides were placed in 100% acetone, then in carbol-xylol, then in 100% xylol, followed by 100% xylol, then balsam and cover glass.

The slides were allowed to remain in each solution down to water for about 5 minutes. The time in the 50% alcohol and 95% alcohol solutions need be only 3 minutes. The dilution of the alcoholic fast green staining solution with acetone slows down the action and makes it easier to use.

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[2]McWhorter, F. P. and Weier, E. 1936. Possible uses of dioxan in botanical micro-technic. Stain Tech . 11, 107–117.

[3]Safranin O, (not certified), from G. G übler and Company, Leipzig, Germany.

[4]Fast green F. C. F., (not certified), from Michigan Biological Supply Company, Ann Arbor, Michigan.

A TRIPLE STAIN FOR AMPHIBIAN EMBRYOS

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Methods commonly employed for staining early amphibian embryos are in certain respects inadequate. With iron hematoxylin it is notably difficult to stain nuclei to the proper intensity without at the same time overstaining the yolk granules. In addition, most counterstains generally used after Delafield's or similar hematoxylins, fail to produce color differentiation between yolk granules and ground cytoplasm. In attempting to stain salamander embryos (*Triturus*), the writers have found a simple tri-stain combination which yields the desired cytological distinctions without loss of clarity in the embryological configuration. This follows the tendency emphasized by Gatenby[1]—"a trend away from these older techniques towards the use of certain methods which give more information about the histology and cytology of development".

The stain combination we suggest involves the successive use of Harris' hematoxylin, safranin, and fast green. The following schedule has been found to be most effective:

1. Fix embryos for six hours or longer with the Bouin-dioxan mixture of Puckett[2] which consists of two parts Bouin's fluid and one part dioxan. (This mixture avoids the shrinkage and hardening usually encountered after other fixatives. Good staining results were also obtained after Zenker, but brittling and shrinkage occurred.)

2. Wash and dehydrate with dioxan; clear for five minutes in xylol to hasten infiltration; infiltrate and imbed in hard paraffin (56–58 C.). Section and mount as usual.

3. Stain for five minutes in Harris' hematoxylin[3] (National Aniline Co., FH-12) and wash in water. Destain with 35% acid alcohol and blue in tap water.

4. Stain for five minutes in 1% safranin O (National Aniline Co., NS-10) made up in aniline water, and wash in tap water.

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[1]Gatenby, I. B. 1928. *In* Lee's Microtomist's Vade-mecum, *9th Ed.*, 279. P. Blakiston's Son & Co., Philadelphia, Pa.

[2]Puckett, William O. 1937. The dioxan paraffin technic for sectioning frog eggs. Stain Techn., **12**, 97–98.

[3]For reference purposes the manufacturer's name and the certification numbers of the batches of stains employed by the writers are given here.

5. Counterstain for one to two minutes with 0.5% fast green (National Aniline Co., NGf-3) made up in 95% alcohol. This step is critical. Control timing so that yolk granules remain bright red while ground cytoplasm is stained green. Wash and dehydrate with absolute alcohol.

6. Clear with oil of cloves or xylol and mount in balsam.

A successfully stained preparation shows the resting nuclei blue against the green of the ground cytoplasm. Chromosomes in mitosis appear purplish and the spindle fibers are sharply defined in green. Yolk granules and nucleoli are red; therefore the former will contrast nicely against the ground cytoplasm. All components are well balanced in intensity and sharply defined one from another. The stains used in this combination also possess the advantage of permanency.

THE USE OF BISMARCK BROWN Y IN SOME NEW STAIN SCHEDULES[1]

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ABSTRACT.—The staining quality of Bismarck brown Y may be improved and sterility maintained by adding 5% phenol to a 1% aqueous solution. Use the phenolic Bismarck brown in combination with iron alum hematoxylin except for stripped epidermis in the following procedures:

Stem and Root Schedule: Mordant sections from water in 4% iron alum for 10 minutes. Rinse in distilled water and stain in 0.5% aqueous hematoxylin for 1 minute or until darkly stained. Rinse in distilled water and destain in 2% iron alum until a gray color appears. Rinse thoroly in distilled water and intensify hematoxylin by transferring sections to 0.5% aqueous lithium carbonate until the desired black color appears. Rinse thoroly in distilled water and stain for 1–5 minutes in phenolic Bismarck brown. Rinse in distilled water, dehydrate successively in 30, 50, 70, 95 and 100% alcohol. Clear in methyl salicylate for 5 minutes, then to xylene for 3–5 minutes, and mount in balsam.

Middle Lamellae in Wood: Destain more thoroly in 2% iron alum than for the general stem and root schedule, and intensify in lithium carbonate for a longer period (about 1 hour).

White Potato Tuber Sections: Modify above schedule by reducing time of destaining in 2% iron alum to about 30–60 seconds and intensify hematoxylin until starch grains appear bluish in color. Stain in phenolic Bismarck brown for 1–2 minutes.

Wheat Grain Sections: Fix grain for sectioning when in "dough" stage. Use schedule the same as for potato tuber except for reducing time of staining in phenolic Bismarck brown to about 45 seconds.

Tradescantia zebrina Epidermis: Strip epidermis from leaf while submerged in water. Fix in 100% alcohol 10 minutes, pass thru 95, 70, 50, 30, and 10% alcohol to water. Stain in phenolic Bismarck brown for 10–20 minutes. Dehydrate, clear in methyl salicylate and mount in balsam.

Bismarck brown Y (anilin brown) merits a wider use as a plant tissue stain. Harrar (1928) has recommended Bismarck brown for phloem. It is also useful for staining many other tissues. In combination with iron alum hematoxylin, clear differentiation is obtained

[1]Papers from the Department of Botany, The Ohio State University, No. 417.

and the stains are relatively permanent. Tissues stained with this combination photograph quite satisfactorily. As a plant histological stain, Bismarck brown Y[2] produces better results in an aqueous solution than in alcohol; but after an aqueous solution has stood for a week or ten days at ordinary laboratory temperatures a fungus often develops in it, necessitating frequent filtering. In an effort to prevent growth of this fungus and thereby eliminate the need of frequent filtering, alcohol, toluene, formalin and phenol were added in various concentrations, as antiseptics, and tests were made as to the staining properties as compared to the dye when made up in distilled water. Transverse sections of *Menispermum canadense* stems were used as test material. All sections were cut 26 μ in thickness and carried thru the same stain schedule. It was found that alcoholic solutions as dilute as 20% were effective in preventing growth of the fungus, but the stain produced was inferior to that produced by other solutions. Four drops of toluene added to 50 cc. of an aqueous solution of Bismarck brown kept it sterile and the staining reaction appeared to be the same as for the pure aqueous solution. When the dye was made up in 5% formalin, growth of the fungus was prevented and a superior staining reaction resulted. The same was true for 5% phenol solution. The phenol solution was regarded as being somewhat superior to the formalin solution in differentiation and definition. This stain solution is referred to in the following schedules as *phenolic Bismarck brown Y*. The formula is as follows:

Bismarck brown Y (anilin brown Y).....	1 gram
Phenol crystals.....	5 grams
Distilled water.....	100 cc.

Mix the constituents, allow to stand for 1 hour, then filter. The solution is then ready for use and will keep indefinitely. Recently the writer discovered that Simmons (1886) over 50 years ago used carbolic acid in Bismarck brown as a stain for leaf tissues, epithelial cells and bacteria.

Hematoxylin with Phenolic Bismarck Brown Y for Herbaceous and Woody Stems and Roots

1. If sections are in 95% alcohol transfer at 3 minute intervals through 70, 50, and 30% alcohol to distilled water.

[2]The original experiments with Bismarck brown Y were made in 1933 with uncertified dye produced by Coleman and Bell, Norwood, O. More recently certified Bismarck brown Y, No. CN 1 and CN 3, produced by the same manufacturers have produced almost identical results. Dr. Elizabeth Wagner aided the writer in some of the original experiments.

2. Transfer sections to 4% aqueous solution of iron alum (ferric ammonium sulfate) for 10 minutes.

3. Rinse with 6 changes of distilled water to remove excess mordant (iron alum).

4. Decant the water and cover sections with a 0.5% aqueous solution of hematoxylin[3] for 1 minute or until they are dark colored.

5. Rinse in 2 changes of distilled water to remove excess hematoxylin.

6. Decant the water and cover sections with a 2% solution of iron alum. This destains and removes undesirable hematoxylin precipitate. Permit the destaining to continue until the tissues have a grayish appearance. A few trials likely will be necessary before the right point is reached.

7. To stop the destaining action decant the iron alum and rinse 6–8 times in distilled water.

8. Intensify the remaining hematoxylin by transferring the sections to a 0.5% aqueous solution of lithium carbonate.[4] The time required ranges from a few seconds to an hour, depending upon the amount of hematoxylin remaining on the cell walls and the intensity desired. The black color produced by action of the lithium carbonate on the hematoxylin is quite desirable because of the increased differentiation.

9. Rinse in distilled water 2–3 times to remove excess lithium carbonate. If this is not thoroly removed, a flocculent precipitate appears when the sections are placed in the phenolic Bismarck brown.

10. Decant the water and cover the sections with phenolic Bismarck brown Y. The time of staining varies with different structures and the degree of intensity preferred. Usually from 1–5 minutes is sufficient.

11. To stop action of the phenolic Bismarck brown Y decant the stain and rinse the sections with distilled water.

12. Decant the water and dehydrate the sections by an alcohol series consisting of 30, 50, 70, 95 and 100% at 3 minute intervals. If the tissues are imbedded in collodion, the 100% alcohol may be dispensed with by the use of ether-absolute-alcohol (half and half

[3]Camp's (1930) method of preparing aqueous hematoxylin is very satisfactory. Distilled water is brought to the boiling point. The heat source is removed, then the hematoxylin (light) crystals are added. The solution is ready for use as soon as it is cool. A trace of sodium bicarbonate added to the solution improves the immediate staining qualities.

[4]A 0.5% lithium carbonate solution in distilled water is an excellent reagent for intensifying hematoxylin in many tissues. For some species, however, it has a macerating effect on cortical tissues. This has been noted for young and mature stems of *Helianthus annuus*, 3–5 year old stems of *Asimina triloba*, *Saururus cernuus* stems and the fruiting tips of *Fucus vesiculosus*. Sometimes the macerating effect may be removed by diluting the lithium carbonate. This dilution, of course, slows up the intensifying action on the hematoxylin. Sodium carbonate, used in the same way as the lithium, occasionally has the same macerating effect. If these solutions cannot be used, alkaline tap water or dilute ammonia may be employed.

mixture) which will dehydrate the tissues and remove the collodion at the same time. Make 2–3 changes.

13. Transfer the sections to oil of wintergreen (methyl salicylate) for 5 minutes.

14. Transfer the sections to xylene for 5 minutes or longer, then mount them in Canada balsam.

Staining Schedule for Middle Lamellae in Wood

Excellent preparations demonstrating middle lamellae may be made from fresh or dried wood of many species. *Asimina triloba* and *Pinus strobus* are very satisfactory forms with which to work. Small wood blocks stored for 1–3 weeks in glycerine with 95% alcohol (half and half) will section satisfactorily without imbedding, on a sliding microtome. Air in the wood blocks should be removed by pumping when first placed in the glycerine-alcohol mixture, or by boiling in water for a few minutes, then plunging in cold water, repeating this process until sinking occurs. Then boil again and plunge the blocks immediately in the glycerine-alcohol mixture. The blocks may be left in this preservative indefinitely. After 2–3 years, however, white pine shows some deterioration. Occasionally cells may have the primary and secondary wall pulled loose from bordering lamellae.

The *hematoxylin phenolic Bismarck brown Y schedule for stems and roots*, with the following modifications, is excellent for sections of wood to demonstrate middle lamellae:

1. Destain more thoroly with the 2% iron alum solution, or until a light gray color is obtained.
2. Rinse in distilled water 6 times.
3. Transfer to 0.5% lithium carbonate solution for 30–60 minutes, or until the middle lamellae appear as black lines.
4. Continue as for the regular stem and root schedule.

A Modified Staining Schedule for White Potato Tuber Sections

The hematoxylin phenolic Bismarck brown Y combination is especially good for differentiating starch grains, protein crystals and internal phloem in the white potato (*Solanum tuberosum*) tuber sections. When properly stained the starch grains will show a bluish color similar to that produced by iodine. The striations and hilum are satisfactorily differentiated. Cubical protein crystals may be found in cells a short distance (3–5 cells) inside the cork layer. With the following staining schedule these crystals stain light brown to a red brown color. Only occasional tubers have an abundance of these crystals. Potato tuber pieces will imbed and section satis-

factorily from collodion, using Wetmore's (1932) method. For staining, modify the stem and root schedule as follows:

1. Destain slightly in the 2% iron alum, 30–60 seconds usually being sufficient. The sections after destaining should have a dark gray color.
2. Intensify the hematoxylin with 0.5% lithium carbonate solution until the starch grains show a bluish color.
3. Stain in phenolic Bismarck brown for 1–2 minutes.
4. Finish as in steps 12, 13 and 14 of the *stem and root schedule.*

Stain Schedule for Wheat Grains

Wheat grains for sectioning should be collected when in the "dough" stage. With a sharp razor blade slice off a tip of each grain to aid entrance of the fixative. Fix in a chromo-acetic acid solution using Schaffner's, Nawaschin's or the Chicago formula. Removal of air with a filter pump aids in better fixation. Wheat grains prepared in this way may be imbedded in collodion and sectioned satisfactorily. Wetmore's (1932) hot collodion method is very satisfactory. Use the same schedule as for potato tubers except that care must be taken *not* to overstain in the phenolic Bismarck brown. Usually 45 seconds is sufficient time for this stain. When properly done the very delicate endosperm cell walls will appear as fine brown lines, the starch grains a bluish black color, irregular dark masses of protein may occur along with the starch grains, aleurone cells will be filled with brown protein granules and the nuclei are stained dark blue.

Preparation and Staining of Epidermal Tissue for Permanent Mounts

1. Allow *Tradescantia zebrina* (Wandering Jew) leaves to wilt for 4–6 hours.
2. Strip off epidermal layers, keeping the leaf submerged in water during the process. This prevents serious rolling of the tissue.
3. Fix the strips in absolute alcohol for 10 minutes.
4. Transfer at 3 minute intervals through 95, 70, 50, 30 and 10% alcohol, then into distilled water.
5. Stain in phenolic Bismarck brown for 10–20 minutes.
6. Rinse in distilled water.
7. Dehydrate at 3 minute intervals in 10, 30, 50, 70, 95 and 100% alcohol.
8. Transfer to oil of wintergreen (methyl salicylate) for 10 minutes or longer.
9. Cut the strips into ⅛ inch squares with a sharp safety razor blade.
10. Transfer to a drop of balsam on a slide, being careful to carry over as little wintergreen oil as possible.
11. Examine with a microscope to see that the outer surface of the epidermis is turned up. Then cover with a cover-glass.

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

BOOK REVIEWS

BENSLEY, R. R. and BENSLEY, S. H. **Handbook of Histological and Cytological Technique.** 8½ x 11 in., 167 pp. Cloth, with ring binding. Univ. of Chicago Press, Chicago, Ill. 1938. $2.00.

This book is the outgrowth of the authors' laboratory instruction leaflets, and the material given in it has previously been available in mimeographed form. Probably it will prove of limited usefulness in other than the authors' own classes. It contains 30 pp. of "Techniques for the study of fresh tissues" and about 130 on "Techniques for the study of fixed tissues." Unfortunately formulae for staining fluids and other solutions are often indefinite; it is not specified whether chemicals are to be used in crystaline or anhydrous form, dye-content of the stains called for is not mentioned, and only in a few instances are the sources given from which the dyes employed by the authors were obtained.—H. J. Conn.

STITT, E. R. CLOUGH, P. W. and CLOUGH, MILDRED C. **Practical Bacteriology, Haematology and Animal Parasitology.** *9th Ed.* 6 x 8½ in., 961 pp. Cloth. 208 illustrations. P. Blakiston's Son and Co., Philadelphia, Pa. 1938. $7.00.

One must take the authors' word that this is "9th edition", altho the book comes out under a new title, with additional authors, in a new format, and is completely rewritten. In other words, it is virtually a new book, 124 pages longer than the 8th edition. For bibliographic purposes, the change from the old title is a little unfortunate; the 8th edition was "Practical Bacteriology, Blood work and Animal Parasitology" by E. R. Stitt. The new edition is improved in appearance because of wider margins; this makes the page larger, altho the type-page has not been changed in size.

This book is too well known to need many words of description. It is especially to be noted, however, that the material on apparatus, culture media and staining methods, formerly comprising the first three chapters of the book, have now been transferred to the appendix. This appendix is now over 100 pages long and constitutes a concise manual of bacteriological methods. The fact that methods of staining and preparing culture media have been transferred to it does not mean that they have been slighted by the authors. On the contrary the formulae have in many cases been brought up to date, the less useful methods omitted and more workable procedures inserted. A table of the solubilities of bacteriological stains is included.—H. J. Conn.

MICROSCOPE AND OTHER APPARATUS

BOETGEHOLD, H. **An improvement of the field of view in microscope objectives.** *Zts. wiss. Mikr.*, 55, 17–25, 1938.

By the use of suitable menisci the author attempts to correct for the curvature of the field of view as is corrected by a homal in photography. The refractive index of such a lens should be positive or only slightly negative. For an objective of medium power a single thick meniscus at the top is sufficient. Its concavity is far greater than its convexity, and is directed away from the object. For a higher power objective a smaller but stronger lens is used at the lower end, its concavity toward the object.

Since these menisci have the chromatic error of converging lenses, a series is used at the top of the high power objective. This requires the use of compensating oculars.

Planachromatic lenses for oil immersion can be made of 1.00 N.A.; with proper choice of dye and use of monochromatic light this becomes equivalent to 1.25 N.A.—*E. Barasch.*

HANSEN, W. W. **On a new design of micromanipulator.** *J. Royal Micro. Soc.*, **58**, 250–2. 1938.

This plan relates to the development of a new type of micromanipulator capable of allowing motion of a quartz needle, or other fine tool, for small distances and in any direction. The conditions attained are: considerable ratio between the motion of the operator's hand and the needle, good rigidity, freedom from backlash, freedom from wear, and ease of construction (no close fits required). On a test for possible lost motion it is found that the average difference in micrometer settings amounts to about one wave length of light.—*J. A. de Tomasi.*

MACNEAL, W. J. and BLEVINS, A. **A culture slide for dark-field microscopy.** *Science*, **88**, 554. 1938.

The slide (Arthur H. Thomas Co., Philadelphia, Penn.) has a central circular stage about 3 mm. in diameter which is surrounded by a circular moat approximately 0.9 mm. deep and 4.5 mm. wide at the top. The wall slopes away from the central stage so that the stage is at the upper surface of a truncated cone with a broad base. A second narrow and shallow groove entirely encircles the moat, slightly separated from it. The former is used for the culture medium, the smaller groove catches the excess vaseline or paraffin used for sealing the cover slip to the slide.—*J. A. de Tomasi.*

MAYNARD, C. W. **An efficient paraffin bath.** *Am. Jour. Clin. Path., Tech. Suppl.* **3**(2):73–75. 2 figs. 1939.

Lamps, wired in parallel, dip into drinking glasses containing paraffin and paraffin diluted with chloroform. Heating is controlled by using appropriate size bulbs. Tissues in perforated containers rest on the unmelted paraffin. Here the temperature cannot be much above the melting point of the paraffin.—*G. H. Chapman.*

MILLS, JOHN H. **A multiple ring maker for the Kline test.** *J. Lab. & Clin. Med.*, **24**, 189–191. 1938.

The Kline test is a microscopic precipitation test for laboratory diagnosis of syphilis, performed by mixing 0.5 cc. of heated serum and about .008 cc. antigen emulsion on a glass slide and observing with 100–120 times magnification. Usually four rings are made for each test: unknown serum plus diagnostic antigen; unknown serum plus exclusion antigen; known positive serum; known negative serum.

A rigid brass multiple ring maker is suggested to reduce time required setting up the Kline test. Cut 12 one-inch lengths of brass tubing 16 mm. outside diameter and about 1.2 mm. wall thickness. Solder these to a brass plate, about $\frac{1}{8}$ inch thickness, cut to same dimensions as slides used for test. Space is saved if rows are somewhat staggered. Dress ends of tubes with a file and polish on glass surface sprinkled with emery powder. A bakelite valve handle may be fastened to the top of the brass plate by a screw, making an instrument resembling a rubber stamp. Small vents are drilled thru the plate at the center of each tube. Further helpful suggestions are made regarding the use of this instrument in Kline diagnostic and exclusion tests.—*P. R. Beamer.*

STUDNIČKA, F. K. **A container for the staining of microtome sections placed on glass.** *Zts. wiss. Mikr.*, **55**, 43–44. 1938.

In order to accommodate slides of other than the usual size of 2.6×7.6 cm and more of them without injuring freshly prepared sections to be stained and washed, the author devised the following vessels: (1) A crystallizing beaker, diam. 18 cm., with a vertical wall at least 7.6 cm. high (i.e., length of slides). (2) Another beaker to be placed inside it, diam. 15 cm., perpendicular walls 7.6 cm. high. (3) A third beaker of any height to cover the first two like a lid.

Place the slides in the staining fluid between (1) and (2), the section facing the concavity of the outer wall, and other slides in water in the central beaker for washing.—*E. Barasch.*

PHOTOMICROGRAPHY

WOLF, J. **A new method of marking certain areas in a section.** *Zts. wiss. Mikr.*, **55**, 44–48. 1938.

A method of placing an easily recognizable, easily removable mark on the area of a section to be photographed without injuring the slide or impeding its examination has been proposed by the author. A transparent tape with a cross mark is placed on the cover glass. It consists of (1) transparent, sticky Durex cellulose tape, 19 mm. wide, (Durex Abrasives Corporations, 82 Beaver St., New York), the sticky layer consisting of gum and resin; (2) thin (0.14 mm.), transparent, cellulose tape, 24 mm. wide, with circular holes, diam. 10–12 mm., 5–6 mm. apart; (3) an ordinary thick, square or round cover glass, 18–20 mm. wide, bearing two diagonal lines, drawn with a lithographic pen, crossing in the center of the cover slip.

Place tape (1) on tape (2), leaving only the perforations sticky. Place a cover slip on each 3rd perforation; divide the tape, leaving one marked cover slip in the center and one perforation on each side. Place cross mark on the area to be studied and press it down on sticky holes holding it in place by a clip. Find area in field and focus for photography. Remove tape.—*E. Barasch.*

WYCHERLEY, S. R. **Photomicrography and record photography with Dufaycolor.** *J. Royal Micro. Soc.*, **58**, 244–9. 1938.

After a general discussion of the several color materials available at the present for photographic purposes, this paper describes the nature of the film in the Dufaycolor process, in which the so-called reseau, or color mosaic, is an integral part of the sensitive emulsion. It is pointed out that the fineness of ruling (40 lines to the mm.), together with the high order of transparency of the dyes used, combine to produce a medium peculiarly suited to the needs of scientific photomicrography. The transparency of the film gives ready-made lantern slides. A set-up for this technic, as suggested, may require: 5-amp. white arc at 24 in. from substage, 6-in.-focus collecting lens, Dufay compensating filter, Abbe condenser, ¼ in. objective (N.A. 0.8, W.A. approx. 0.5), × 8 eyepiece. The magnification is × 200, the exposure for a medium-stained specimen 1/10 sec. Full instructions for processing the film are also appended.—*J. A. de Tomasi.*

MICROTECHNIC IN GENERAL

AUMONIER, F. J. **Notes on the distortion of paraffin sections.** *J. Royal Micro. Soc.*, **58**, 253–7. 1938.

Graphic reconstruction of skull development from paraffin ribbons has shown a considerable degree of distortion of serial sections even after spreading. The question which the author seeks to answer is: do the sections, always creased and wavy, regain their full length? Of the many factors that have a bearing on this problem the following are studied: consistency (and melting point) of paraffin, shape of block, sharpness of knife, and angle of blade. It is found, by comparing paraffins of 52° and 58° C. m. p., that low melting point paraffin is preferable. The sharpness of the blade is the limiting factor, i. e. it must be given the greatest care. Variations in the angle of the blade between 5° and 17° do not seem to cause appreciable difference of distortion. As distortion in paraffin is permanent, it is suggested that, whenever possible, celloidin, or double imbedding should be used.—*J. A. de Tomasi.*

GARVIN, T. **The use of dioxan in histologic technique.** *Amer. J. Clin. Path., Tech. Suppl.*, **2**, 175–8. 1938.

An outline of the history, chemistry and toxicology, and use of dioxan. Fix small fragments, over a low flame until vapors arise, in the following solution: 40% formalin, 10 cc.; dist. water, 90 cc. Fix larger sections not more than 2 mm. thick 1–2 hr. at 60–65° C. Transfer to the following baths: 95% alc., 1 hr.; pure dioxan, 2 changes of 1 hr. each; dioxan-paraffin (1:1), 1 hr.; paraffin, 2 changes of 15–30 min. each. Embed, cut, mount and stain as with paraffin.—*G. H. Chapman.*

PORTER, C. L. **Bound water as an aid in the preparation of microscopic mounts.** *Turtox News*, **17**, 14. 1939.

Methods of mounting microscopical preparations which do not lend themselves to the balsam technic (e. g., glycerin jelly or Venetian turpentine) often fail to yield permanent or reliable mounts. The behavior of gelatin, which retains moisture indefinitely in the presence of formalin, has suggested the following mounting technic: Transfer material from water to a very dilute gelatin. Allow gradual concentration until a fairly firm jelly is formed (the slower the process, the less tendency there will be for the material to plasmolyze). Transfer a small portion of the jelly containing the material to a glass slide, warm gently until it melts, and apply a cover slip. Place 1–2 drops of formalin at the edge of the cover slip; let stand several hours to allow diffusion of the formalin; blot and wipe dry. Ring the mount with gold size, lacquer, or varnish.—*J. A. de Tomasi.*

DYES AND THEIR BIOLOGICAL USES

ABRAHAMSON, E. M. **The determination of blood glucose with 2,6 dichlorphenolindophenol.** *Am. Jour. Clin. Path., Tech. Suppl.*, **3**, 60–4. 1 Fig. 1939.

The Folin-Wu filtrate is reduced by ferricyanide in alkaline soln., acidified, and the ferrocyanide titrated with 2,6 dichlorphenolindophenol using a soln. prepared from a standard tablet marketed by Hoffman-LaRoche. This indicator is added from a burette until the pink color of the dye persists.—*G. H. Chapman.*

JOHNS, C. K. **Place of the methylene blue and resazurin reduction tests in a milk control program.** *Amer. J. Pub. Health*, **29**, 239–47. 1939.

For routine testing the methylene blue reduction test has many advantages over the usual counting methods. Its accuracy as an index of the initial bacterial content of the better grades of milk is greatly improved when the organisms are redispersed by inversion of the tubes every 2 hours. The resazurin 1-hour test does not indicate the bacterial content as reliably as does the modified (tube inversion) methylene blue reduction test, the former placing a larger proportion of high count milks in the highest grade. When incubation is continued to the pink stage, the accuracy of the resazurin test compares favorably with that of the methylene blue test, while the reduction time is shortened. The correlation between resazurin color and cell count is much poorer with market milks than with individual quarter samples. Many market milks with high cell counts fail to bring about a significant color change.—*M. W. Jennison.*

PEARLMAN, M. D., and LIMARZI, L. R. **Correlation studies of basophilic aggregation and reticulocytes in various clinical conditions.** *Amer. J. Clin. Path.*, **8**, 608–19. 1938.

The basophilic aggregation test of McCord is shown to be another, but inferior, method for staining reticulocytes and is, therefore, an index of marrow response rather than an indication of lead absorption.—*G. H. Chapman.*

TOBIE, W. C. **Improved procedures in determination of aldehydes in distilled alcoholic liquors with Schiff's reagent.** *Food Res.*, **3**, 499–504. 1938.

The writer suggests certain refinements in technic to avoid some of the difficulties experienced in using Schiff's fuchsin-sulfurous-acid reagent for the colorimetric determination of aldehydes. An entirely colorless reagent may be prepared by treatment with decolorizing carbon: Dissolve 0.500 g. basic fuchsin in 500 cc. warm water, and pass in sulfur dioxide gas until the weight has increased by 5 g. Make up to 1 litre. If still yellow or orange in color, add 1 g. of decolorizing carbon, agitate, and filter with a watch glass covering the filter. Repeat this treatment if any discoloration remains after first treatment. This reagent may be kept 5 months or more in a well-stoppered container. An adjustment for sensitivity is frequently necessary because of variations in dye content of different lots of fuchsin; the solution should show a distinct, altho light, pink color with 0.20 cc. of the dilute standard acetaldehyde as given in the A.O.A.C. method, while the aldehyde-free check is colorless or nearly so. If this does not prove to be the case, adjustment can be made by passing in a little more SO_2 or dissolving in the solution small quantities (e.g. 0.05 g.) of basic fuchsin, until the desired sensitivity is reached. The writer also discusses the preparation of

aldehyde-free alcohol and describes methods of conducting the determination.—*H. J. Conn.*

ANIMAL MICROTECHNIC

BEYER, E. M. **Double imbedding method for rubber paraffin.** *Amer. J. Clin. Path., Tech. Suppl.*, **2**, 173–5. 1938.

The method is useful for blood vessels, endocrine glands, gastro-intestinal tract and for large sections. It permits cutting 5 μ sections without fraying. Heveatex, a natural rubber latex, is spread in a thin film on a glass slide. After drying 24 hr. it is pulled off and cut into small pieces. To 100 g. of paraffin add 2 g. of rubber and 0.5 g. of beeswax. Heat to 105° C. for 16 hr. with occasional stirring. Avoid overheating.

For visceral and neural tissue, employ the following schedule: 10% formalin, 24 hr.; 80% alc., 24 hr.; 95% alc., 24 hr.; abs. alc., 24 hr.; abs. alc. and ether, 24 hr.; 4% celloidin, 24 hr.; abs. alc. and ether, 1 hr.; xylol, 15–30 min. or until transparent then 3 changes of 56–58° C. rubber paraffin in oven for 1½–3 hr. Embed in rubber paraffin as with paraffin.—*G. H. Chapman.*

CROSSMON, GERMAIN C. **Separation of the acidophilic elements of the tissues into two groups.** *Anat. Rec.*, **73**, 163–170. 1939.

Zenker-fixed sections stained with acid fuchsin and immersed in an aqueous phosphotungstic or phosphomolybdic acid solution exhibit two classes of tissue elements: Class 1, those which retain the acid fuchsin (cytoplasm, muscle, erythrocytes, zymogen, etc.); Class 2, those decolorized in the solution (collagen, reticulum, cartilage, mucin, etc.). Class 2 structures are those elements which ordinarily are stained blue in Mallory's connective tissue stain.

Fluorescein dyes may be used instead of acid fuchsin previous to immersion in the acid solution. Class 1 elements then retain a red color; Class 2 elements are orange. Class 2 elements are subsequently decolorized completely in abs. alcohol. They are then generally remordanted in phosphotungstic acid and stained in light green or anilin blue and then differentiated in 1% acetic acid.

This gives three classes or elements ordinarily taking acid dyes. Class 1A (red): erythrocytes, acidophilic granules, zymogen, keratinized epithelium, etc.; Class 1B (lightly stained green or blue): general cytoplasm, smooth and skeletal muscle; Class 2 (green or blue): collagen, reticulum, cartilage, bone, etc. Fifteen steps in making the staining solutions and applying the stain are given in the paper.—*S. I. Kornhauser.*

FAZIO, C. **Betrachtungen über die histologischen Methoden mit Benzidin im Zentralnervensystem.** *Zts. gesam. Neurol. u. Psychiat.*, **164**, 678–686. 1938.

The effect of post mortem changes on Pickworth's benzidine method for blood capillaries (*J. Anat.* **69**, 62–71, 1934; *Abs., Stain Tech.* **11**, 132, 1936) was studied. Rabbits, white mice, guinea pigs, and dogs were used. The author concludes that the method gives valid results up to 48 hr. post mortem in material stored at room temperature or to 5 days when stored in a refrigerator.—*H. A. Davenport.*

GERMAN, W. M. **Hortega's silver impregnation technique, uses and application.** *Amer. J. Clin. Path., Tech. Suppl.*, **2**, 165–71. 1938.

An introduction to Hortega's methods, including general directions, and details for Hortega's general nuclear stain, and the stain for epithelial fibrils, epithelial bridges and protoplasmic reticulum. No new methods given.—*G. H. Chapman.*

GERMAN, WM. M. **Hortega's' silver impregnation stains.** *Am. Jour. Clin. Path., Tech. Suppl.*, **3**, 47–55. 4 Figs. 1939.

Detailed instructions are given for: (a) Hortega's neuroglia stain, which provides an excellent contrast between black stained neuroglia cells and other tissues, stained brown or mahogany. Microglia fibrils are not stained; (b) Hortega's stain for microglia which colors them brown or mahogany red. They can be recognized by a polymorphic nucleus, an irregular cell body and cell processes; (c) Hortega's impregnation method for certain epithelial neurofibrils and cell processes not demonstrated by ordinary methods; and (d) Hortega's modification of the silver tannate method of Achucarro, which demonstrates mitochondria,

cellular fibrils and certain cell processes not seen by ordinary methods.—*G. H. Chapman.*

GERMAN, W. M. **Hortega's silver impregnation methods. Technique and applications.** *Amer. J. Clin. Path., Tech. Suppl.*, **3**, 13–9. 1939.

Details are given for the double impregnation method for connective tissue, which is useful for demonstration of some of the finer connective tissue fibrils in connective tissue, inflammatory tissue, sarcomata of fibroblastic origin, and the fibrillar structure of nerve sheaths. The fibrils stain deep brown to black while epithelial cells stain red or golden brown. The double impregnation method with $KMnO_4$ is useful for staining all connective tissue elements, particularly the finer fibrils which are not seen by ordinary methods. It demonstrates nerve sheath structures and neurofibrils. Gallego's general tissue stain colors nuclei, magenta; epithelial cytoplasm, yellowish pink; connective tissue, brilliant green; muscle, olive green; and cornified epithelium and blood, grass green. Gallego's method for differential staining of elastic tissue gives excellent differentiation between elastic and collagenous connective tissue and is useful for photomicrography. The former stains a brilliant fuchsin red, while the latter stains a brilliant green.—*G. H. Chapman.*

HUMPHREYS, S. P. **A method for the impregnation of perivascular nerves on intracerebral blood vessels.** *Am. J. Path.*, **15**, 151–153. 1939.

Method A. Human material previously fixed in formalin. Dissect out the vessels and wash 12 hr. in dist. water. Soak in the following mixture 4 hr.: 95% alcohol, 90 cc.; glacial acetic acid, 5 cc.; formalin, 5 cc. Wash 2 hr. in dist. water. Impregnate 6 to 24 hr. in Bodian's silver nitrate copper solution—1 to 2% aq. protargol, (Winthrop Chemical Co., New York City) 20 cc.+2 g. metallic Cu (Merck). Wash in 3 changes of dist. water. Reduce with: hydroquinone 5 g., sodium sulfite, 10 g., dist. water 100 cc. Tone in 1% yellow $AuCl_3$ solution, wash in 2 changes of dist. water, treat with 2% oxalic acid solution+1% formalin until specimens are light blue, wash and fix with "hypo". Dehydrate with 95% and abs. alcohol and clear with oil of bergamot. Flatten the vessels and mount in balsam. Use dissecting microscope for the original dissection and flattening of the vessels.

Method B. Fresh tissue from animals. Perfuse the brain with physiological saline solution immediately after death till the blood is removed, then inject 500 cc. of the alcohol, acetic acid, formalin mixture (formula same as that used in method A). Remove the tissue and fix in the same solution 24 hr. Dissect out the blood vessels and wash and stain as in method A.—*H. A. Davenport.*

MOLLIER, G. **Eine Vierfachfärbung zur Darstellung glatter und quergestreifter Muskulatur und ihrer Beziehung zum Bindegewebe.** *Zts. wis. Mikr.*, **55**, 472–73. 1938.

This quadruple staining method gives excellent differentiation for the study of connective tissue muscle relationship. Practically any fixing fluid suitable for the "Azan" technic may be used for fixation.

(1) Transfer sections from 70% alcohol to Orcein (Tänzer-Unna) for 12 hr. (2) Rinse thoroly in dist. water until no color discharges. (3) Slightly overstain in Weigert's iron-haematoxylin, 1–3 min. (4) Rinse in dist. water. (5) Differentiate briefly in 1% HCl in 70% alcohol until nuclei are distinct, extracting excess orcein simultaneously. (6) Wash in tap water for 15 min. (7) Apply azocarmine G. 15–30 min., cold. Do not overstain. (8) Rinse in dist. water. (9) Transfer to 5% phosphotungstic acid, 3 changes for 2–6 hr. until collagen fibers are completely decolorized. (10) Rinse in dist. water. (11) Napthol green 15–30 min. (1 g. napthol green B (Hollborn) in 100 ml. dist. water plus 1 ml. glacial acetic acid). (12) Transfer directly to 95% alcohol (keeping sections in motion to prevent precipitation of the green) ½ min. (13) Run thru abs. alcohol, xylol, balsam. If elastic tissue stain is to be omitted begin at step 3.

Results: Chromatin and nucleoli stain pure deep blue; cytoplasm, bluish-red; myofibrils, deep red with sharp contours; elastic fibers, brown-black; mature collagen fibers, olive green; erythrocytes, brilliant red.—*J. M. Thuringer.*

RALSTON, MARTHA B., and WELLS, ARTHUR H. **Azocarmine stain for bone marrow.** *Am. Jour. Clin. Path., Tech. Suppl.*, **3**, 72–3. 1939.

Azocarmine, in combination with blue dyes, is most satisfactory for differential

staining of bone marrow. It gives vividly contrasting colors of myeloid elements and has the advantage that the cells are left in their *in vivo* relationships. The technic is as follows: Decalcify medullary bone over night in 5% acetic acid in Zenker's solution; wash 24 hr. in tap water; and prepare in paraffin by usual methods. Cut 4 μ sections, remove paraffin with xylene, and run thru alcohols to water. Heat azocarmine solution (Grubler's azocarmine, 1 g.; dist. water, 100 cc.: when dissolved add 1 cc. glacial acetic acid) in a paraffin bath at 56° C. Immerse slides 20 min. Wash in several changes of dist. water. Decolorize and differentiate in aniline alcohol (aniline, C.P., 2 drops; 95% alcohol, 50 cc.) followed by acid alcohol (95% alcohol, 100 cc., glacial acetic acid, 2 drops) until sections are rose red (30–90 sec.). Wash in dist. water. Stain 10–15 sec. in: 0.5% toluidine blue (C.I. 925), filtered just before use. Wipe off excess water. Dehydrate quickly in abs. alcohol. Modify slightly for different batches of stain.—*G. H. Chapman.*

SHERMAN, L., and SMITH, L. W. **Notes on the rubber paraffin method for embedding tissues.** *Amer. J. Clin. Path., Tech. Suppl.*, 2, 171–3. 1938.

Peel sheets of pale crepe rubber (Quaker City Rubber Company, Philadelphia) into many layers and shred finely. To 160 g. of melted 53° or 58° C. paraffin at 78° C. add 20 g. (5 g. every 12 hr.) with frequent stirring until the colorless mixture is homogenous (about 4 days). Store at 55–60° C. Discard when the rubber separates out. For embedding, add 12.5 g. (varied according to desired consistency of block) to 500 g. of 53° or 58° C. paraffin and 5 g. of beeswax or bayberry wax at 60° C., stirring occasionally. It hardens and keeps well at room temp. (The stock mixture will not keep this way). Put back into the oven 24 hr. before use. The method is useful for making paraffin sections in hot weather, and particularly for eye sections and embryos. Standard dehydrating and clearing technics can be used.—*G. H. Chapman.*

PLANT MICROTECHNIC

GAUTHERET, R. **Sur la possibilité de realiser la culture indefinie de tissus de tubercules de carotte.** *Comp. Rend. Acad. Sci.*, 208, 118–120. 1939.

Small portions of carrot tissue may be kept alive indefinitely in the following: Distilled water, 500 cc.; Knop solution, 500 cc.; mineral solution of Berthelot, or yeast ashes, 0.3 g.; glucose, 20.0 g.; gelatin, 13.0 g.; vitamin B_1, 0.001 g.; cysteine hydrochloride, 0.01 g.; indol B. acetic acid, 0.00001 g.

The cultures should be kept in diffuse light and free from necrotic areas and roots. Transplants should be made about every two months.—*E. Weier.*

KUWADA, Y., SHINKE, N., and OURA, G. **Artificial uncoiling of the chromonema spirals as a method of investigation of the chromosome structure.** *Zts. wiss. Mikr.*, 55, 8–14. 1938.

By the use of a solution causing an alkaline reaction in plant spermatocytes, leaf epidermal nuclei, and pollen mother cells of *Tryxalis nasuta, Spiranthes australis, Impatiens Balsamina, Tradescantia reflexa,* etc., an attempt was made by the authors to dissolve the chromosome matrix and follow the steps in the process of uncoiling of chromonema spirals. Cells treated in this way previous to the usual acetocarmine stain show solid chromosome masses, irregularly drawn out spirals, and other stages of haplomitosis in much more detail than do ordinary preparations. The following chemicals were used: ammonia vapor, NH_4OH (1/100 mol), NH_4Cl (0.5 mol), NH_4SCN (0.5 mol), $(NH_4)_2SO_4$ (0.25–0.5 mol), KCN (2^{-7}, 2^{-8} mol), KOH (0.003 mol), NaOH (0.003 mol), NaCN (2^{-6}, 2^{-7} mol), Na_2HPO_4 (0.2 mol), NaOCl (2^{-6} mol), $NaHCO_3$ (2^{-8} mol).—*E. Barasch.*

LEITNER, J. **The use of acetocarmine in the study of the contents of mature, completely dried pollen grains.** *Zts. wiss. Mikr.*, 55, 48–50. 1938.

Pollen grains are noted for retaining their germinative power a long time. The author shows the presence of one, two, or three cells within pollen grains as much as 112 years old. Due to the hardness of some of the husks, the grains could not be crushed and emptied. This was remedied by soaking them a few minutes in acetocarmine on a slide. The outlines of the sperm cells soon become recognizable. In 24 hr. they are as distinct as in fresh pollen grains. After breaking the husks, the staining time is no longer than with fresh grains. Species of the

families *Labiatas, Liliacea, Ranunculacea,* etc. were used for experiment.—*E. Barasch.*

LEFÈVRE, JOSEPH. **Similitude des actions cytologiques exercées par le phenylurethane et la colchicine sur des plantules végetales.** *Compt. Rend. Acad. Sci.*, **203**, 301–304. 1938.

Seeds of wheat, either before or during germination, were treated for 48 hr. at room temperature with vapors of phenylurethane, or with a saturated solution diluted 15 to 20 times. This treatment resulted in hypertrophy of the coleoptile, mesocotyl and root growing point, and finally in arrested growth. Mitosis was blocked and large nuclei with many chormosomes were formed.—*E. Weier.*

MICROÖRGANISMS

LIPP, J. **Detection of Spirochaeta pallida.** *Munch. Med. Woch.* **86**, 294. 1939.

A method is described for the detection of *Spirochaeta pallida* in primary lesions. Rub primary lesion with either swab and platinum loop or dry muslin until a clear serous exudate appears. Make a thin smear of the serum, dry in air (avoid heat or fixatives), stain 3–4 minutes with 3% aq. Victoria blue, wash, dry, and examine under oil immersion. The organisms appear sharply defined and stained a deep blue.—*L. Farber.*

RYO, EIHYO. **A simple method of staining bacterial flagella.** *Kitasato Arch. Exper. Med. (Tokyo).*, **14**, 218–220. 1937.

Smears from a young culture, dried and fixed as usual, are stained for 3–5 min. at room temp. in a combined mordant and stain prepared as follows. Soln. 1: 5% phenol, 10 cc.; powdered tannin, 2 g.; sat. soln. potas. alum, 10 cc. Soln. 2: sat. alc. soln. of crystal violet. For use, 10 parts of Soln. 1 and 1 part of Soln. 2 are thoroly mixed at the moment of staining. After staining the solution is washed off, and the preparation mounted in cedar oil. The solutions, before mixing, can be preserved a long time.—*H. J. Conn.*

T'UNG, T. **Photodynamic action of safranine on Gram-negative bacilli.** *Proc. Soc. Exp. Biol. & Med.*, **39**, 415–7. 1938.

Safranin exhibited a photodynamic action on Gram-negative organisms. Previous experiments with other dyes failed with this group of organisms. Sat. aq. safranin O (water-soluble, National Aniline and Chemical Co.) was diluted in water adjusted to pH 8.0 with NaOH. Organisms (22 strains of various species) were mixed with dye and exposed to light for 2 hr. The maximum action was obtained with 1% safranin. Both weaker and stronger concentrations were less effective. Unexposed cultures were in most cases uninjured by safranin alone. The photodynamic action as such was increased in experiments in which 0.15% H_2O_2 was used. It is suggested that oxidation may play a rôle in photodynamic action.—*M. S. Marshall.*

HISTOCHEMISTRY

CARERE-COMES, O. **New methods for the histochemical demonstration of potassium, and selective staining of tissues rich in potassium.** *Zts. wiss. Mikr.*, **55**, 1–6. 1938.

The author finds that sodium paradipicrylamine sold as "siena orange" by Dr. K. Hollborn & Sons, Leipzig S3, ready for use, stains tissues selectively and quantitatively for potassium, by the following technic (1) Treat sections that have been fixed in neutral formol solution and embedded in paraffin as follows: remove paraffin, place in 100%, 90%, 70% alcohol, dist. water, (2) Siena orange solution, 2 min.; 10% HCl, 3 min.; wash in twice renewed dist. water, 10 min.; dry at 37°C. or with filter paper and cover with thickened cedar oil. The tissues containing potassium stain orange; others stain pale yellow, or not at all.

Contrasting stain for muscle fibers: repeat (1) above, place in siena soln., 2 min.; 10% HCl, 3 min.; aq. acetone, 5–15 min.; aniline blue for contrast (0.01% aq. soln. to be freshly prepared from 1% stock soln.), 10–15 min. Wash in dist. water, dry with filter paper, clear with abs. alcohol (quickly) or oil of cloves and xylol, and embed in neutral Canada balsam (Hollborn) or thick cedar oil. The orange muscle fibers show up well.

Contrasting stain for hemopoietic organs: (1) as above; place in siena orange solution, 1 min.; 10% HCl, 3 min.; dist. water, twice renewed, 10 min.; differentiate carefully with dil. acetone, 5–10 min.; stain with gentian violet (10 drops of sat. alc. soln. in 20 cc. dist. water), 2 min. Wash with dist. water and dry with filter paper. Differentiate carefully in abs. alcohol. Clear with xylol, embed in neutral Canada balsam. Erythrocytes appear orange.—*E. Barasch.*

KELLEY, E. G. **Reactions of dyes with cell substances. V. Differential basic combination of tissue nuclei with special reference to resting and mitotic cells of tumor tissue.** *J. Biol. Chem.*, **127**, 73–86. 1939.

This method has been developed in an attempt to indicate the amount of nucleic acid in the nucleus. Nuclei with greater nucleic acid content bind more basic dye at the same pH. The complete staining of the nucleus is the point of measurement recorded. For example, the nuclei of thymocytes have less nucleic acid than the nuclei of lymphocytes; nuclei in mitosis more than resting nuclei.

Acid and basic dyes were made up at a concentration of 0.1% of actual dye by dissolving weighed amounts of the dye in 50 cc. portions of buffer solutions. Thionine was used in Kolthoff's borax-succinic acid buffers from pH 3.0–5.8, also in dilute HCl and acetate buffers to cover range from pH 2.0–6.0. Toluidine blue was soluble in all the buffers, but was used mostly in Sörensen's citrate HCl and NaOH buffers to cover the range from pH 2.0–7.0. Fast green was soluble in all buffers. All dyes were from certified batches. Buffers were made up at 0.2 pH-intervals and their pH-values determined to ± 0.02 pH by means of a vacuum tube glass electrode. Likewise the dye solutions were tested to ± 0.02 pH. Tissues were fixed in: alcohol (strength not given); alcohol containing 5% acetic acid; mercuric chloride at pH 2.0 and 6.0; phosphotungstic acid at pH 2.0–6.0. Sections from material were embedded in paraffin, cut at 7μ, and passed from xylol thru alcohols to distilled water. Individual slides were transferred for 15 min. to a 35×80 mm. glass shell vial containing 50 cc. of a buffer solution of same pH as acid and basic dye solutions . They were then rinsed in distilled water; transferred to 0.1% dye-buffer solution for 1 minute; rinsed in distilled water; transferred to 0.1% acid dye-buffer solution for 1 minute; rinsed in distilled water; dipped in 95% alcohol until no further running of dye could be observed; placed in 100% alcohol; and mounted in balsam, after passing thru xylol. Sections were compared with a comparision microscope. A Wratten filter (B2) is recommended for better comparison.—*J. E. Kindred.*

KELLEY, E. G. **Reaction of dyes with cell substances. IV. Quantitative comparison of tissue nuclei and extracted nucleoproteins.** *J. Biol. Chem.*, **127**, 55–71. 1939.

Extraction of nucleoproteins from three different types of rat tissue (sarcoma, carcinoma, and thymus gland) shows that substances of constant composition can be obtained from each tissue. Each tissue yields materials with the same ratio of nucleic acid to protein, and the same isoelectric point. Toluidine blue in aqueous solutions of 0.1% and 0.005% is also used as a means of testing the similarity of tissue nuclei and extracted nucleoproteins. These tests show a complete lack of correlation between the isoelectric point of tissue and that of the extracted protein. These experiments show, however, that in the case of alcohol-fixed tissues the amount of dye taken up follows very closely the relative nucleic acid content of the nucleoproteins in the nucleus. This cannot be said about $HgCl_2$ or formalin fixation.—*J. A. de Tomasi.*

LANCASTER, SARAH. **Nature of chromaffin nerve cells in certain annulates and arthropods.** *Trans. Amer. Micr. Soc.*, **58**, 90–6. 1939.

Neither methylene blue, used as a vital stain, nor $FeCl_3$, NaOH, $CuSO_4$, or KCN gave indication, by precipitation tests, of an adrenalin-like substance in living giant nerve cells of earthworms, grasshoppers, or leeches. In all cases the cords were fixed in Orth's fluid, stained with Heidenhain's Fe alum hematoxylin, and Gage's acid fuchsin as a counterstain. In the same organisms, however, giant chromaffin nerve cells were found that gave positive reactions to the chromate tests for adrenalin. In fixed tissues, the chromaffin reaction made the cytoplasm appear denser than that in the surrounding regions. Findings support the assumption that these giant chromaffin nerve cells possess dual properties of nervous and adrenal cells, and may represent the origin of both the sympathetic nervous and the adrenal systems in vertebrates.—*J. A. de Tomasi.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the April number of this Journal.

STAINS CERTIFIED MAR. 1, 1939 TO MAY 31, 1939*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Orange G	CO–6	78%	As counterstain in histology	Mar. 3, 1939
Eosin Y	DE–3	81%	For use in bacteriological media	Mar. 18, 1939
Basic fuchsin	DF–5	93%	For use in bacteriological media	Mar. 29, 1939
Methyl violet 2B	NMv–6	87%	As histological and bacteriological stain	Mar. 30, 1939
Brilliant green	NBg–8	89%	For use in bacteriological media	Apr. 25, 1939
Giemsa stain	GGe–6	....	As blood stain	May 10, 1939
Giemsa stain	NGe–5	....	As blood stain	May 22, 1939
Cresyl violet	NW–7	93%	For use in histology	May 22, 1939
Neutral red	NX–7	65%	As histological and vital blood stain	May 25, 1939

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

STAIN TECHNOLOGY

VOLUME 14 OCTOBER, 1939 NUMBER 4

A NEW METHOD OF SILVER IMPREGNATION FOR NERVE CELLS AND FIBERS

H. R. DEBAUCHE, *Biological Laboratory, University of Louvain, Belgium*

ABSTRACT.—**Fragments of tissue, immediately after death, are fixed in Debaisieux's modification of the Duboscq-Brazil picro-acetic-formol fluid, and treated as follows: Hydrate by soaking 2–6 hr. in distilled water with 30 drops of conc. NH_4OH per 100 cc. Freeze and cut sections about 25 μ in thickness. Bleach sections about 15 min. in ammoniacal water (52 drops conc. NH_4OH per 100 cc. water). Transfer to 20% $AgNO_3$ solution and heat at 45° C. till light brown. Add conc. NH_4OH drop by drop till the Ag precipitates and then redisolves into an opalescent solution. Pour solution and sections into a little distilled water and transfer sections quickly to formaldehyde solution (3 cc. formalin to 100 cc. water). Dip sections in distilled water and transfer to 1% aqueous gold chloride till deep blue. Place for about 10 minutes in 5% aqueous sodium thiosulfate solution for fixing and clearing. Wash thoroly in tap water, dehydrate and mount. Special directions are given for applying this technic to delicate material such as insects, and for use with serial sections.**

This method and its application on free sections has recently been published.[1] An application of this method to sections on slides will be given hereafter.

The method is based on the same principle as Dr. Dewulf's recently published discovery,[2] i.e. the use of a nascent silver complex instead of the stable combination. We have preferred, however, in the place of Hortega's sodium carbonate, to use pure ammonia for the precipitation of the silver nitrate as in Rogers' method, the impregnations thus obtained being much more energic and complete, especially where invertebrate material is concerned.

[1]Debauche, H. R. 1939. Nouvelle méthode d'imprégnation des cellules nerveuses par le nitrate d'argent. Ann. Soc. Sci. Bruxelles. Ser. II, 59, 23–27.

[2]Dewulf, A. 1938. Une nouvelle méthode d'imprégnation des neurofibrilles. Journ. Belg. Neurol. Psychiâtric. 1938, No. 6, 407–410.

The age of the animal is unimportant. The fragments of tissue to be impregnated must be fixed immediately after death. The best results are obtained with material fixed in Debaisieux's modification of the Duboscq-Brazil[3] fluid, i.e.:

Ethyl alcohol 80%	100 cc.
Picric Acid	2 g.
Acetic Acid	10 cc.
Formaldehyde	40 cc.

Good results have also been obtained on material fixed in formaldehyde (Merck's pro Analysi) 10 to 20%.

It is advisable to use only the purest obtainable chemicals; Merck's "Pro Analysi"[4] products have given satisfying results.

The material must be preserved in the fixing fluid as long as convenient. A long fixation will give the best results. It has been demonstrated that, in material fixed for a short time only, the background of the preparation shows a tendency to take on the impregnation, obscuring more or less the finest details. This tendency disappears gradually by lengthening the period of fixation. A fixation of 100 days will give very clear preparations; splendid results were obtained with material fixed and kept in Duboscq-Brazil fluid for over one year.

Before sectioning, the pieces are soaked in distilled water with 30 drops of conc. ammonia per 100 cc. This hydrating process must last from two to six hours according to size of pieces.

While this hydration is in progress, prepare the following solutions:

1. *Ammoniacal water:* Distilled water . . . . . 100 cc.
   Ammonia (Merck's pro analysi) . . 52 drops.
2. *Silver nitrate:* Distilled water . . . . . 20 cc.
   Silver nitrate (Merck's pro analysi) . . . . 4 g.
3. *Formaldehyde solution for reduction:*
   Formaldehyde (Merck's pro analysi) . . 3 cc.
   Distilled water . . . . . 100 cc.
4. *Yellow gold chloride:* 1% aqueous.
5. *Sodium hyposulfide* (sodium thiosulfate): 5% aqueous.

When the pieces are sufficiently hydrated, proceed as indicated below:

a) Section by freezing technic (25 μ has proved to be the most convenient thickness).

[3]Debaisieux, P. 1935. Organes scolopidiaux des pattes d'insectes. I. Lépidoptères et Trichoptères. La Cellule, 44, 273–314.

[4]This correspondes to the "reagent" grade of the American company of the same name.

b) The sections are received in a dish containing the ammoniacal water. They are left to bleach in this bath for about 15 minutes.
c) Transfer the sections to the silver nitrate solution, in an Erlenmeyer flask.
d) Continually agitating, heat moderately (45° C.), till the sections become light brown.
e) Always agitating, precipitate the silver nitrate slowly in the solution containing the sections, by addition of pure ammonia one drop at a time. Then redissolve the precipitate carefully by adding more ammonia. This process must be stopped when the stage of opalescence is attained.
f) When this stage is reached, pour the solution with the sections immediately into a dish containing a little distilled water.
g) Transfer the sections rapidly to the formaldehyde solution, where the reduction takes place. A good reduction is recognized by the uniform, rich brown color which the sections take in this solution.
h) The sections are then dipped in distilled water and transferred immediately to the gold chloride solution. They are left in this bath till their color changes to a deep blue.
i) Transfer the sections to the sodium thiosulfate solution where they are fixed and cleared, about 10 minutes. Then wash abundantly in tap water.
j) Dehydrate and mount in neutral Canada balsam, with cover glass.

If the impregnation has been correctly performed, the nerve cells and intraprotoplasmatic neurofibrillae appear dark blue on a pink, almost colorless ground. The nuclei are transparent, well defined.

For delicate material, such as insects, of which frozen sections are very difficult to obtain, it is generally better to proceed as follows:

a) Place pieces 24 hours in: Alcohol 90%.....50 cc.
Conc. ammonia.....15 drops.
b) One hour and a half in absolute alcohol.
c) 20 minutes in toluol.
d) Six hours in paraffin with 20% beeswax, in the oven (58° C.).
e) Imbed in same paraffin.
f) The sections are cut as usual with paraffin imbedded material, but the ribbon is transferred directly in xylol which dissolves the paraffin, thus freeing the sections.
g) The sections are transferred into absolute alcohol, to which distilled water is added, very carefully and slowly, one drop at a time. Water must be thus added until all alcohol has been removed and

the sections are thoroly hydrated. The impregnation can then be carried on as on frozen sections.

Application of the Method to Serial Sections on Slides

The material must be fixed in Debaisieux's modification of the Duboscq-Brazil fluid. The pieces are imbedded in paraffin as indicated above. The sections must be carefully spread on the slide, to ensure sufficient adherence.

a) The paraffin is removed by xylol and the sections are hydrated, using only chemically pure alcohol.
b) The slide is brought into ammoniacal water (distilled water 50 cc.; conc. ammonia 26 drops), and left in this bath for about 10 minutes.
c) Dip slide in distilled water and transfer into a 10% solution of silver nitrate. This bath is heated to 45° C. The slide is left in this solution about 10 minutes, till the sections become light brown.
d) Transfer the slide immediately to a silver ammoniacal complex prepared in advance. This solution is prepared as in Roger's method, but using only a 10% solution of silver nitrate. The slide is left in this bath from 1–5 minutes.
e) Dip slide in distilled water and transfer immediately to reducing bath, i.e.: 3% solution of pure formaldehyde.
f) Tone in gold chloride (1%).
g) Fix in 5% solution of sodium thiosulfate.
h) Wash well in tap water.
i) Dehydrate and mount in neutral Canada balsam, with cover glass.

This method has been successfully tried on man, rabbit, rat, different birds and numerous invertebrates, especially insects.

A QUADRUPLE STAIN COMBINATION FOR PLANT TISSUES

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ABSTRACT.—**A new procedure for the quadruple staining of paraffin sections of plant tissues is offered, involving the use of safranin, methyl violet, fast green and orange G. Differentiation is practically automatic and is controlled by the use of special washing solutions. Striking color-contrast effects permit the identification of every type of cell structure and cytoplasmic inclusions.**

Quadruple stain combinations are still comparatively new in botanical microtechnic. One was devised by Dr. Geo. H. Conant (outlined in *Triarch Topics* for March, 1938), but the writer found that the excessive use of clove oil solutions in this procedure was objectionable for the reason that it is very difficult to remove absolutely every trace of the clove oil, with the result that fading sets in sooner or later. An effort was therefore made to devise a different procedure using the same dyes, and also to minimize as far as possible the personal judgment factor.

The dyes used in quadruple staining include safranin O, fast green FCF, methyl violet 2B and orange G. A fifth stain can, if desired, be added in the form of Harris' hematoxylin; this is used before the other stains and should not be differentiated as far as usual since it will become somewhat weakened during the differentiation of the other dyes. Crystal violet may be substituted for the methyl violet, and gold orange for the orange G.

The disadvantage inherent in any quadruple staining procedure is that washing solutions become saturated with excess stain after only a few slides have been passed thru them. Water alone cannot be used for all the washes; otherwise those stains already applied would be removed or their intensity considerably lessened. The necessity for frequently replacing washing solutions would make any procedure unduly expensive; consequently it is desirable to employ relatively inexpensive reagents for removing excess stain solutions.[1] Given an inexpensive washing reagent, it must then be one which does not have any effect upon the affinity of any dye for the structures which

[1]Of the solvents used in the present work, the methyl cellosolve was a Carbide and Carbon Chemicals Corp. product and the tertiary butyl alcohol was obtained from the California Botanical Materials Co. The prices are respectively $2.40 and $2.10 per gallon; the cheaper USP clove oil is entirely satisfactory, and with 95% ethyl alcohol costing about 56 cents per gallon, the washes are not unduly expensive.

it is supposed to stain, but it should remove the dye from all other structures for which it normally has no affinity and leave them clear for staining by other dyes. On the other hand, the staining solutions must have their staining capacities enhanced by the addition of other reagents; in other words, any dye which is more of a general than a selective stain must have its affinity accentuated. Overlapping or mixing of stain affinities is very undesirable. When used on plant tissues, safranin, for example, tends to be more of a general than a selective stain and therefore requires the addition of other substances to cause it to become more readily differentiated.

Only tissues in which there is a variety of cell types or considerable chemical difference in the structure of the cells and the cytoplasmic inclusions are adaptable for quadruple staining. Relatively undifferentiated tissues merely show the general effect given by a Flemming triple combination. If no starch is present, for instance, there is little reason for using the violet dye, but it must be mentioned that starch grains and plastids have been found in totally unexpected places as a result of applying quadruple combinations.

The procedure, as finally revised after trial on a wide variety of plant tissues, is as follows. The solutions are somewhat complicated, but the actual procedure is simple.

1. Bring slides bearing paraffin sections down to 70% ethyl alcohol.

2. Stain in safranin O for 24–48 hours. Overstaining is rarely possible except with certain Gymnosperm structures. With the latter, the time should be reduced to 2 hours. To make the safranin solution,[2] dissolve 4 g. certified safranin O dye (the writer used a National Aniline and Chemical Co. sample, No. NS-10) in 200 cc. methyl cellosolve (ethylene glycol monomethyl ether), then add 200 cc. 50% ethyl alcohol, 15 cc. formalin and 3 g. sodium acetate. The formalin acts as a mordant, the sodium acetate accentuates the brilliancy of the dye, and together they permit an unusually sharp differentiation.

3. Rinse in tap water. This step should not be omitted, otherwise the dye may become irremovably precipitated.

4. Stain in 1% methyl violet 2B (a National Aniline sample, Cert. No. NMv-5, was used) for 10–15 minutes.

5. Rinse in running tap water.

6. Rinse for 5 seconds in a mixture of equal parts of 95% ethyl alcohol, methyl cellosolve and tertiary butyl alcohol.

[2]Johansen, D. A. 1938. Tertiary Butyl Alcohol methods. IV. Hygrobutol methods. El Palo Alto News 2: 1–4.

7. Immerse for 10 minutes in a fast green FCF solution prepared as follows: take one part of a saturated solution of the dye in equal parts of clove oil and methyl cellosolve, 3 parts 95% ethyl alcohol, 3 parts tertiary butyl alcohol, and add 3–4 drops of glacial acetic acid to each 100 cc. of solution. The dye (a Coleman and Bell product, purchased before certification was instituted, was used) should be dissolved in the solvent several days before being used; fast green usually dissolves slowly—an iridescent streaming first occurs and solution is not complete until this streaming has disappeared. If the acetic acid is omitted, the dye will be completely removed during succeeding stages. The time may require some adjustment according to the nature of the tissues and the fixing fluid. That cited is for formalin-aceto-alcohol and Navashin fixation; incidentally, the writer has found that the substitution of propionic acid for the acetic acid in these two fixing solutions gives far superior fixation.

8. Rinse for 5 seconds in a mixture of equal parts of 95% ethyl alcohol, methyl cellosolve and tertiary butyl alcohol plus a trace of acetic acid.

9. Immerse for about 5 minutes in the orange G stain. First prepare a saturated solution of the dye in equal parts of clove oil and methyl cellosolve, then take one part of this mixture and one part each of methyl cellosolve and 95% ethyl alcohol. (The dye originally used was a National Aniline product, Cert. No. NO-3.)

10. Rinse briefly in a wash composed of equal portions of clove oil, methyl cellosolve and 95% ethyl alcohol.

11. Rinse in a wash made up of equal parts of clove oil, absolute ethyl alcohol and xylol. (If Nevillite V is used for mounting, substitute toluol for the xylol.)

12. Rinse in xylol (or toluol) plus a trace of absolute ethyl alcohol.

13. Rinse in two changes of pure xylol (or toluol), then mount in dammar balsam, Nevillite V or other preferred medium.

The staining solutions can accommodate upwards of 500 slides (when held in Coplin jars) before replacement is required, but the washing solutions in steps 6, 8 and 10 will need changing frequently —usually after about every 50 slides. As soon as one of them becomes too heavily saturated with dye, it should be renewed; otherwise the sharpness of the stains will be diminished.

The slides when taken out of the first three staining solutions will be rather dirty and no differentiation is apparent, but when removed from the wash in step 10, they will be perfectly clean, provided Haupt's adhesive has been used for affixing the sections. If the periods as cited for each staining solution are adhered to, there is no

need for the exercise of personal judgment; differentiation is practically automatic. The staining process is continuous once the slides have been removed from the safranin.

The staining effects are little short of astonishing. Dividing chromatin is red, resting chromatin purplish; the nucleoli are usually red, occasionally violet, but in *Lilium* megagametophytes, which are exasperatingly difficult to stain adequately, they are a bright green. Nucleoplasm is colorless or greenish. Spindle fibers are purple. Lignified cell walls are a bright red, cutinized cell walls reddish purple, suberized walls red, cellulose walls greenish orange, and the middle lamellae green. Sclereids are a brilliant red; collenchyma cells are a bright green, or yellowish green when not fully developed. The cytoplasm is normally a bright orange, but when the combination is used on roots or rhizomes showing the origin of lateral roots, the cytoplasm of the latter is a dull green and the nuclei are purplish. Starch grains have a deep purple center with a green, and then an orange, halo at the periphery, but the two latter colors are unfortunately somewhat fugitive and become bleached or replaced by the purple. Plastids are yellowish-green to bright green. The callose portion of the guard cells of stomata should be bright red and the remainder of the cell wall purplish. Casparian strips are dark red and the rest of the cell walls of the endodermis yellow. Invading fungal mycelium is bright green; the combination, in fact, is unusually good for sections of the teliosori and uredosori of *Puccinia*. Sections of lichens take an excellent stain, and the algal and the various fungal regions are well differentiated. Since only killing fluids giving an acid fixation image have been used, the staining reactions of mitochondria are not known.

DELAFIELD'S HEMATOXYLIN AND SAFRANIN FOR STAINING MERISTEMATIC TISSUES

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ABSTRACT.—**The following technic is suggested for staining permanent preparations of meristematic tissues: Prepare and mount the sections by the usual paraffin method. From water, stain them 2-10 minutes in a solution made by adding 2-4 cc. of Delafield's hematoxylin to a Coplin jar full of tap water. As staining is progressive, the sections should be examined from time to time with a microscope. When the cell walls have become a deep purple, transfer the preparations, thru the usual series, to a mixture of xylol-absolute-alcohol in equal parts, and from this to a counterstain made by adding 4-6 cc. of a saturated solution of safranin in absolute alcohol to a Coplin jar full of xylol (75%) with absolute alcohol (25%). This stains the nuclei. Leave the sections in the counterstain at least 2 hours and then rinse them in xylol-absolute-alcohol (1:1) to remove excess safranin. Transfer them to pure xylol and then mount them in neutral balsam.**

The recent revival of interest in histogenetic problems in plants has created a need for better methods of staining the cell walls and nuclei in meristematic tissues. Altho several excellent technics have been devised for this purpose (Foster, 1934; Cross, 1937), the differences in the staining reactions of different species, and of a given species when different fixatives are used, are such that a universal method seems very remote. Difficulties encountered in obtaining satisfactory preparations of the shoot apex in certain species of *Acacia* (Boke, 1939) stimulated experimentation leading to a technic which involves the use of Delafield's hematoxylin and safranin. The present method of applying these well-known stains has yielded such excellent results with *Acacia*, *Ginkgo*, *Tradescantia*, and *Zamia*, that it seems worth while to call it to the attention of investigators who are interested in histogenetic problems. Moreover, it requires less time than the conventional methods.

Altho the best results were obtained when Juel's reagent (prepared according to a formula given by Korody, 1937) was used as a fixing medium, other good preservatives may be used. Sections are prepared by the usual paraffin method and mounted on slides. The following is a provisional schedule for staining, which may be modified according to the needs of the user:

1. Xylol—5 to 8 minutes.
2. 50% xylol–50% absolute alcohol—2 minutes.
3. Absolute alcohol—2 minutes.
4. 95% alcohol—2 minutes.
5. 70% alcohol—2 minutes.
6. 50% alcohol—2 minutes.
7. Water—2 minutes. Rinse several times.
8. Delafield's hematoxylin—2–10 minutes [prepared by adding 2–4 cc. of a stock solution, made according to a formula given by Chamberlain (1932), to a Coplin jar full of tap water]. This stains the cell walls an intense purple. Since there are always two variables, the concentration of the staining medium and the material being stained, the precise time must be determined by trial. Staining is progressive, however, and gradual enough so that the preparations may be examined from time to time with the aid of a microscope; this permits the intensity to be easily regulated. Depending upon the composition of the tap water used in its preparation, the staining medium is more or less stable. Its staining powers gradually decrease, owing to a deposition of pigment on the walls of the container. It has been found, however, to remain usable for two weeks or more.
9. Water—2 minutes. Rinse several times.
10. 70% alcohol—2 minutes.
11. 95% alcohol—2 minutes.
12. Absolute alcohol—2 minutes.
13. 50% xylol–50% absolute alcohol—2 minutes.
14. Safranin—2 hours (prepared by adding 4–6 cc. of a saturated solution of safranin in absolute alcohol to a Coplin jar full of a mixture of 75% xylol and 25% absolute alcohol. The resulting staining solution should be a deep red, but clear. If a precipitate forms, it should be filtered before using; it may be kept stable by the occasional addition of a few cc. of absolute alcohol to replace that which evaporates.) This differentiates the nuclei. With some materials, a period longer than 2 hours may be an improvement. As far as could be determined, meristem sections may be left in this medium indefinitely without being damaged, since nuclei do not overstain in it. If mature tissues occur in the material, lignins and tannins tend to retain too much safranin when the time is lengthened.
15. Destain in a mixture of xylol and absolute alcohol. Usually, a few quick rinses in 50% xylol in absolute alcohol will suffice. However, since the rapidity and extent of destaining depends largely upon the amount of alcohol in the destaining medium, the user may adjust this as he wishes.

16. Xylol—5 minutes.
17. Mount in neutral balsam.

Since the nuclei of many plants stain with Delafield's hematoxylin, the use of safranin as a counterstain may frequently be omitted. In this case, the time required for the entire staining process is shortened to less than an hour. It seems, however, that the safranin has a slight tendency to reduce the hematoxylin which is retained by the nuclei, so that the use of the counterstain may yield a preparation with more desirable contrasts. If the tissues do not readily take either the safranin or the hematoxylin, the use of a 1% solution of potassium permanganate as a mordant for 5 minutes previous to staining is suggested (Chamberlain, 1932).

Altho this method of using Delafield's hematoxylin has received mention in the literature (Chamberlain, 1932; Lee, 1937, pp. 159, 652), its value appears to have been underestimated. The method of making up the safranin solution was suggested by a similar procedure used by E. J. Kraus (described by Conant, 1926) who, however, used gentian violet instead of safranin. Cross (1937) and Jones (1939) have likewise used xylol in making up biological stains.

The advantage of the combination is that the preparations are excellent, both for visual work and for photographic purposes. Very satisfactory photomicrographs may be obtained by using panchromatic film and a light green filter.

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DIFFERENTIAL STAINING OF THE ANTERIOR PITUITARY GLAND OF THE CAT

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ABSTRACT.—The terms "acidophile" and "basophile" as applied to the two recognized types of chromophilic cells of the anterior pituitary have been sanctioned by long usage. Nevertheless, these cells do not always give a specific reaction to acid and basic dyes respectively. The difficulty of classification has been further complicated by the demonstration of an additional tinctorial type in the female rabbit and cat which has been provisionally classed as a modified acidophile. This distinction is based on the differential staining reaction obtained with Heidenhain's "azan" technic following sublimate-formalin fixation, the standard acidophile reacting with orange G, the modified type with azocarmine.

Both of these cells are stained with copper hematoxylin, Heidenhain's iron hematoxylin, Mallory's phosphotungstic acid hematoxylin and Kultschitzky's acetic hematoxylin. However, Hansen's chromalum hematoxylin stains the basophiles intensely and also reacts very slightly with the so-called modified acidophile.

The orange G + phosphomolybdic acid + hematoxylin method of Hall and Hunt stains the basophiles more or less selectively when applied progressively, altho the special cells also react. When regressive staining is employed, the results vary depending on the fluid used. Following successful differentiation with Weigert's borax ferricyanide, the basophiles are decolorized and the modified acidophiles are deep blue, while the ordinary acidophiles retain the orange G. These staining reactions are unmodified after Bouin's fixation.

It is concluded that the weight of evidence favors the classification of the special cell as a modified type of acidophile.

INTRODUCTION

The continued interest in the problem of the differential staining of the cellular components of the anterior pituitary is evidenced by the fact that three methods have been described in this journal (Koneff 1938, Maxwell 1938, and Lewis and Miller 1938), since Dawson and Friedgood (1938a) described a technic which differentiated the acidophile of the female rabbit and cat into two distinct tinctorial types. In this method the glands were fixed in sublimate-formol followed by Heidenhain's "azan" modification of Mallory's connective tissue stain. The ordinary or standard acidophile was

colored orange, while the cell which is present during the sexual activity of the female rabbit and cat appeared a deep red due to its special affinity for azocarmine. Material based on this method has been described and demonstrated (Friedgood and Dawson 1938, and Dawson and Friedgood 1937, 1938b).

It has been suggested that the affinity for azocarmine was due to the greater residual mitochondrial content of the cells, since sublimate-formol does fix mitochondria, altho not as well as other fixatives especially designed for this purpose. Others felt that the 'carmine' cell might possibly be an atypically stained basophile. It seemed well to investigate these two possibilities. A variety of staining procedures has been tried on the pituitary of the cat, usually after sublimate-formol fixation, but in one instance Bouin's fluid was used. The relatively high content of acetic acid in this mixture usually destroys the mitochondria, altho this does not always occur.

Specificity of Acid and Basic Stains

The terms 'acidophile' and 'basophile' as applied to the two chromophile cells of the anterior pituitary are sanctioned by usage and probably derived originally from the appearance of these cells after routine staining with one of the common hematoxylins and eosin. Many authors, recognizing that the staining reactions, especially with the anilin dyes, were not always consistent with this earlier distinction of "acidophilic" and "basophilic" types, have preferred to designate the cells by letter or by number so that the implication of a reaction with a specific category of stain could be obviated. It seemed immaterial what system of designation was employed so long as investigators were dealing with well-recognized cell types, but when the possible existence of another type of chromophile is suggested, difficulties of classification immediately appear.

The size and nature of the granules which react with the dyes may also be used to supplement the information obtained by differential staining, but in many instances the appearance of the granulation varies with the fixing fluid used.

Maurer and Lewis (1922) found that the so-called acidophilic cells were stained by safranin, a basic dye, but in the method of Severinghaus, the basophilic cells are stained by methyl green, a basic anilin dye, while in the method of Cleveland and Wolfe (1932) erythrosin, an acid dye, may stain one variety of basophilic cell.

Many of the special hematoxylin stains also behave in an apparently inconsistent manner. Rasmussen's (1936) report, that copper hematoxylin specifically stains the acidophiles but does not stain the basophiles as stated by MacCallum, has been completely

confirmed by Severinghaus (1937) and his co-workers. More recently Hall and Hunt (1938) have described a method employing a simple aqueous hematoxylin, following phosphomolybdic-orange-G and phosphomolybdic acid, which stains one variety of basophile intensely. Warbritton and McKenzie (1937) were fully aware of the inconsistent results obtained by varying fixation and staining methods, and have made a rather complete analysis of the results obtained, but were unable to materially clarify the problem.

The difficulty, moreover, is not confined to the pituitary gland but has its counterpart in other tissues as well. Crossman (1939) has attempted to lay down procedures which will enable the histologist to separate the acidophilic elements of tissues into two groups, based on relative intensity of the acidophilic reaction. Furthermore, similar difficulties have been encountered in finding a stain which is specific for chromatin, and at present chromatin is best identified by a microchemical test (Feulgen's reaction) rather than on the basis of specific staining by recognized basic stains.

Experimental Staining

We may now return to the problem of the basis for recognition of two classes of acidophiles in the cat pituitary or perhaps to the question as to whether the cell type under discussion may be a basophile. The "azan" modification of Mallory's method does not employ a basic dye, and the orange G and anilin blue are sulphonated. After staining with azocarmine, and before differentiation, the special cell is a deep, dark red, the ordinary acidophile, a light red and the dense basophiles, a brilliant red. After differentiation and staining with orange G + anilin blue, only the special cell retains the azocarmine, the ordinary acidophiles are orange and the basophiles blue. When azocarmine staining is omitted both the special cell and ordinary acidophile are orange. However, if anilin blue is present above the optimum concentration, the ordinary acidophile may appear brown due to the addition of anilin blue to the orange G already present. With this combination of stains, so-called selective staining is present to some degree but is relative rather than absolute. However, the final results under a properly controlled procedure are consistent and three selectively-stained cell types are obtained. Nevertheless, the ordinary acidophile may show some affinity for anilin blue and the basophiles first react strongly with azocarmine which is subsequently replaced with anilin blue.

After establishing the tinctorial characteristics of these cells which may also be recognized by differences in form, size, and type of granulation, a variety of hematoxylin stains was applied. With

copper hematoxylin both the special cell and the ordinary acidophile are intensely stained. The special cell is a dull blue, the ordinary acidophile a deep clear blue and the basophiles appear light brown to yellow flecked with incompletely stained mitochondria.

The results obtained by the method of Hall and Hunt are variable depending on the mode of staining. With preliminary staining in orange G followed by a minimum staining in the hematoxylin (3 minutes) without differentiation, the ordinary acidophiles are dull orange, the special cell light to dark brown and the basophiles purple-blue to light blue depending on the degree of their granulation. Both the special cell and the ordinary acidophile tend to react with the hematoxylin but the special cells have a much stronger affinity for this stain. This picture is obtained only if dehydration is rapidly accomplished by passing the slide directly to 95% and absolute alcohol. If dehydration is done slowly thru a graded series of alcohols, the three classes of chromophile cells are a uniform blue color. When the staining in the hematoxylin is more prolonged and differentiation is attempted, the picture changes rapidly. With acid alcohol the blue color is quickly extracted from the basophiles and both the special cell and ordinary acidophile are stained a deep blue. If Weigert's borax-ferricyanide is used for differentiation, the blue color is usually more or less completely extracted from the basophiles and the special cells become a deep blue while the ordinary acidophiles remain orange or light brown. Differentiation with Weigert's fluid is difficult to control and uniform results are not always obtained, but when successful, the differentiation between the special cell and ordinary acidophiles is very striking.

Heidenhain's hematoxylin, Mallory's phosphotungstic acid hematoxylin and Kultschitzky's acetic hematoxylin stain both the special cell and the ordinary acidophile characteristically and with almost equal intensity. Occasionally slight differences in the tinctorial quality of the two cells may be recognized. With Kultschitzky's hematoxylin, when orange G is used as a preliminary stain according to the method of Hall and Hunt (1938), the orange G is replaced but a greenish cast attests to the presence of masked orange G. The basophiles are not stained by these methods. Hansen's chromalum hematoxylin (Bensley and Bensley 1938), if used alone, stains the basophiles a deep blue to purple, while the ordinary acidophiles are colorless and the special cells slightly tinted with blue. When eosin is used as a counterstain, the ordinary acidophiles are brilliant red and the special cells a dull red due to their slight reaction with the hematoxylin.

In order to test the possible effect of the staining of residual mitochondrial material following sublimate-formol fixation, the pituitary gland was also fixed in Bouin's fluid. The azan modification of Mallory's method gave a differential staining similar to that following the sublimate-formol fixation. If anything, the contrast between the azocarmine-stained special cell and the orange-G-stained ordinary acidophile was enhanced—a result similar to that obtained in the rabbit pituitary following fixation in formalin alone. Bouin's fixation, however, like formalin alone, fails to fix the basophiles and chromophobes properly. Accordingly the azocarmine reaction of the special cells cannot be attributed to their greater mitochondrial content; this reaction further indicates that the differential staining with azocarmine and orange G is due to specific differences in the granules of these cells, as revealed by a fixing fluid which contains a relatively high proportion of formalin without bichromate. Furthermore, Bouin's fixation does not modify the staining reactions obtained with the several hematoxylins employed following sublimate-formalin fixation.

The cumulative evidence from differential staining shows clearly that the special azocarmine cell is quite unlike either the standard acidophile or basophile, and its characteristic behavior during the reproductive cycle of the cat affords strong confirmation of its functional independence, altho the significance of its presence and activity is hypothetical at present (Dawson and Friedgood, unpublished). The cell apparently arises from a chromophobe and, when degranulated, returns to a chromophobic state. Moreover, no transition between this cell and the other chromophilic cells has been observed. Whether the special cell is to be classed as an acidophile or basophile is dependent on the relative emphasis placed on the unique specificity of the several stains which have been employed to demonstrate the two classes of chromophiles. My strong inclination, even in the face of some conflicting evidence, as given above, is to regard it as a variety of acidophile.

The suggestion of recognizing a third type of granular cell in the anterior pituitary is regarded by some authors as introducing an unnecessary confusion into a situation which is already too complicated. It also seems unwise to beg the question, however, in the face of the evidence at hand. In many ways there is a close parallel between the cell under discussion and the so-called 'pregnancy cell' which Severinghaus (1937) feels should be regarded as an acidophile in high secretory activity altho it develops from the chromophobes as pregnancy ensues, remains in an active secretory phase thruout

pregnancy and reverts again to the chromophobic state some time after parturition. [Altho these cells are relatively numerous in certain periods of gestation as well as in early postpartum, they cannot be regarded as "pregnancy cells" since they are also found in limited numbers in normal and castrate males, and in proestrous, estrous and castrate females, being typically absent only from anestrous animals.] At the present no final conclusion can be reached as to the nature of the granular content of the azocarmine cell and the matter will have to await the physiological assay of the pituitary gland when these cells are present in great numbers as in late pregnancy.

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PRESERVATION OF TRYPAN BLUE AND NEUTRAL RED WITHIN THE CELLS OF LOOSE CONNECTIVE TISSUE

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ABSTRACT.—**Trypan blue granules within the histiocytes and neutral red stained granules within the mast cells of the same areolar tissue spread can be preserved in their original colored state by the following method: fix in 10% formalin for 12-24 hours; rinse in distilled water; place in two changes of dioxan for 5-10 minutes each (overnight in second change will not hurt); mount from the second dioxan using diaphane dissolved in dioxan as a mounting medium, or clear in xylene and mount in balsam. No dye is lost, and no cellular distortion occurs. Fast green is used as a counterstain (1% aqueous plus 0.2% acetic acid). Stain ½ to 1 minute and rinse.**

INTRODUCTION

This method was devised to make permanent preparations of vitally stained areolar tissue spreads, illustrating both intravital staining by trypan blue and supravital staining by neutral red upon a single slide. A survey of the literature shows that various methods have been employed to preserve each of these dyes when used separately. A critical analysis of the methods used for the fixation of trypan blue was given by Hetherington and Tompkins (1933). They claim that 10% formalin, as used by many investigators, is unsatisfactory when minute amounts of dye must be detected, altho it preserves the cell contours and type of dye deposition. They found that the fixing fluid of Carnoy and Lebrun gave perfect preservation of the dye, but crystals of mercuric chloride caused some interference. Baird (1935) found that 5% neutral formalin followed by acetone dehydration satisfactorily preserved trypan blue in subcutaneous tissue spreads.

Neutral red is more difficult to preserve satisfactorily. A survey of methods used was given by Cunningham and Conn (1932). Following the example set by McJunkin (1925), Zenker-formol has been used extensively as a fixative for neutral red and similar dyes. The problem of dehydration without the use of alcohols was solved by McJunkin and others by employing acetone, followed by benzene or xylene. Hu (1931) found that 100% alcohol, to which $HgCl_2$ and neutral red had been added, permitted complete dehydration without any loss of neutral red from the cells. This method had the further

advantage that paraffin sections of tissue vitally stained by neutral red could be easily counterstained if the dye solution also contained $HgCl_2$ and neutral red. Morrison, Gardner, and Reeves (1936) preserved neutral red in paraffin sections of gastric mucosa by "Susa" fixation, followed by 90% and 100% alcohol, clearing in carbon disulphide, and paraffin embedding. When films can be dried, no dehydration problem is encountered.

Procedure

Modifications of the foregoing methods were tried in order to preserve both trypan blue and neutral red in the same spread of tissue in as nearly as possible their original appearance in the freshly made spread. Various counterstains were tried in order to bring out the

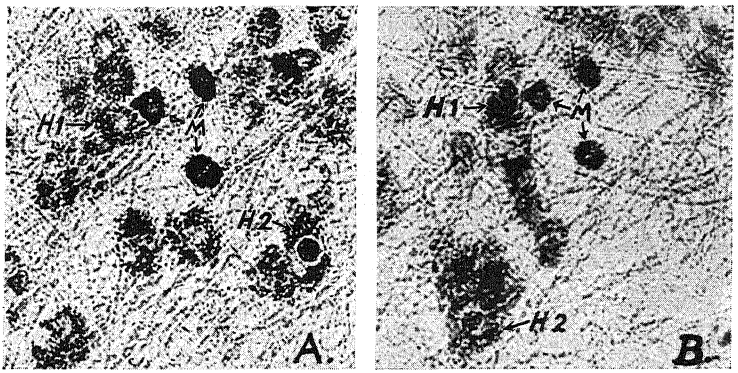

Fig. 1. Identical cells from an areolar tissue spread from a vitally stained white rat, before fixation (A), and after fixation, dehydration, and mounting (B). M, mast cells (neutral red); H1, histiocyte showing trypan blue granules; H2 histiocyte with engorged leucocyte. × 268.

fibrocytes and other tissue elements without altering the vital staining of the histiocytes and mast cells.

A single subcutaneous injection of 2.5 to 3 cc. of a fresh sterile 1% aqueous solution of vital trypan blue (Coleman and Bell Co.) for rats weighing 85–100 g. (5 cc. for mature rats) was sufficient to mark the histiocytes 48 hours after injection. Following light etherization, the animals were killed by severing a large artery. The neutral red solution was injected directly into the subcutaneous areolar tissues of the groin, the needle being directed so as to make several dye deposits around the original puncture. The sample used was certified (NX 5) neutral red, Ehrlich, (79% dye content, National Aniline) 0.02% in 0.9% NaCl. In 3–5 minutes an incision was made and small blobs of the edematous tissue were removed,

placed on clean slides, and teased into thin spreads by the use of needles and small pieces of filter paper. When the corners were dry and attached to the slide, the spread was ready for fresh observation or for fixation.

The most brilliant results were obtained when 10% formalin was used as the fixative. The slight acidity of the commercial formalin is desirable because neutral red acts as an indicator, being red in acid and orange or yellow in alkaline solutions. The 10% formalin should be tested by adding to it a drop or two of neutral red and observing any color change. If it turns orange, cautiously add $N/10$ HCl until it turns red. Fixation is complete in a few hours, but it has been my practice to let the slides fix overnight. Several days fixation in formalin will not hurt the dyes.

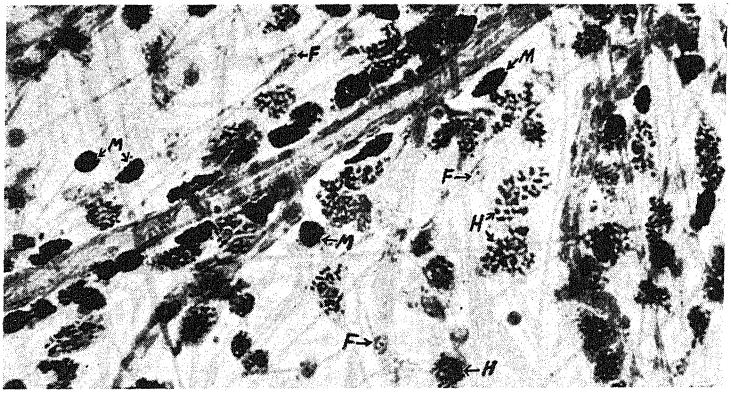

FIG. 2. Permanent areolar tissue spread from white rat intravitally stained by trypan blue, supravitally stained by neutral red, and counterstained by fast green. M, mast cells (neutral red); H, histiocytes (trypan blue); F, fibrocytes (fast green). × 268.

The slides are first rinsed in distilled water and then placed in two successive changes of dioxan (Coplin jars 1 and 2), 3–5 minutes each depending on the thickness of the spread and the amount of agitation. They may stay in the second change indefinitely. The slides are mounted directly from dioxan, using as a medium hardened diaphane (Will Corp.) redissolved in dioxan. They may also be placed in xylene and mounted in balsam. The blue granules within the histiocytes (macrophages) and the fine red granules of the mast cells have very near the same color values as they had in the fresh spreads.

Zenker-formol (85 parts stock plus 15 parts formalin) also preserves the dyes well, altho they will have slightly different color values. $HgCl_2$ crystals will be present to some degree, but 24 hours in dioxan will remove a large proportion of them.

The most useful counterstain tried was fast green F. C. F. (Warner-Jenkinson Co., dye content 85%). A 1% solution in distilled water plus 0.2% glacial acetic acid for ½–1 minute following the rinse after fixation brings out the nuclei, cytoplasmic processes, fibrocytes and fibers. See fig. 2. After staining, rinse in distilled water and place in dioxan.

A few drops (¼ cc.) of Delafield's hematoxylin added to 100 cc. of the 10% formalin fixing fluid stains the nuclei of the spread in 24 hours without interfering with the vital staining. The cytoplasm and fibers of the spread can then be stained by the use of 0.5% aqueous erythrosin (cert. no. CEr-2 Coleman and Bell) for 1 minute.

Summary

By the above methods there is no appreciable loss of either trypan blue or neutral red following fixation, clearing, and mounting. Figure 1, A and B, shows the same cells before fixation, and after mounting. The three mast cells (M) have retained their relative positions, but the histiocytes have migrated, and also do not appear in the same focal plane in fig. 1, B that they had in fig. 1, A. By comparison of the trypan blue granules of histiocyte 1 (H1) in each figure, it is evident that all of the dye is retained.

Slides made four years ago by essentially the same process have retained both dyes without any fading.

The most useful technic employing a counterstain is as follows (see fig. 2):

1. Fixation—10% commercial formalin, 12–24 hours.
2. Rinse in distilled water, 1–2 minutes.
3. 1% fast green plus 0.2% glacial acetic acid, ½–1 minute.
4. Rinse in distilled water, 1 minute.
5. Dioxan 1., 5 minutes.
6. Dioxan 2., 5 minutes.
7. Mount in diaphane dissolved in dioxan.

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A STAINING METHOD FOR THE ANTERIOR HYPOPHYSIS OF THE RAT

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ABSTRACT.—**A staining procedure for the anterior hypophysis of the rat, differentiating between eosinophilic granules, basophilic granules and mitochondria, has been divised. Small pieces of hypophyseal tissue are fixed in Champy's fluid. Following fixation the tissue is either chromated or osmicated. After being embedded in 60–62° paraffin, the tissue is cut serially at 2 and 3 μ. The sections are stained with 7% Altmann's acid fuchsin by heating on a laboratory hot plate, followed by 30 seconds in a 2% solution of Orange G made up in 1% phosphomolybdic acid. They are then treated for 10 seconds in a .01% solution of potassium carbonate, and stained for 10–30 minutes in Goodpasture's acid polychrome methylene blue. The mitochondria stain brilliant fuchsin, the eosinophilic granules orange-red, and the basophilic granules deep blue.**

During the course of recent studies on the anterior hypophysis of the rat, a staining procedure was evolved which satisfactorily differentiates between basophilic granules, eosinophilic granules and mitochondria. The method is somewhat similar to the technics of Severinghaus (1932) and Volkonsky (1932) and was arrived at by making successive modifications of these two technics.

The pituitaries were fixed in Champy's fluid (2 parts of 1% chromic acid, 2 parts of 3% potassium dichromate, and 1 part of 2% osmic acid). Glands weighing up to approximately 10 mg. were divided sagitally into two pieces before fixation; larger glands were usually divided into three or more pieces. In the post-fixation treatment, dehydrating and embedding of the tissue, the methods[1] of Severinghaus (1932) were followed in detail. A majority of the tissue was chromated following fixation (modified Champy-Kull method of Severinghaus). Tissue which was to be used for study of the Golgi apparatus was osmicated following fixation (Nassanov-Kotatchev technic). All tissue was embedded in 60–62° paraffin. Serial sections were cut at 2 and 3 μ; the latter took a more intense stain.

After the sections were run down to water, they were treated as follows:

[1]More detailed directions for the post-fixation treatment and embedding of tissue were kindly furnished us by Dr. Severinghaus in a personal communication.

1. The sections were covered with 1 cc. of 7% Altmann's acid fuchsin. This amount was applied with a pipette so that it covered the lateral two-thirds of the slide. The sections were then heated on a rheostat-controlled laboratory hot plate. In some instances the sections were gently heated to steaming 1–3 times with one minute intervals between the steamings. In most instances less intense heating, without the production of steam, gave better results. Since the hot plate was controlled by a rheostat, it was so regulated that it was hot at one end and cool at the other. Therefore, it was possible to place the slide at different positions to obtain the amounts of heat necessary. The slides were placed on the hot plate at a point where one could barely hold the thumb. They were heated four times for 15 seconds each with a 45 second interval between heatings and a final cooling period of 5 minutes after the last heating. We have found that the reaction of the tissue to acid fuchsin varies from gland to gland and the optimal staining time must be determined for each gland.

2. The acid fuchsin was drained off and the slide rinsed with distilled water. The section was then stained for 30 seconds in a 2% solution of Orange G made up in a 1% solution of phosphomolybdic acid.

3. The slide was rinsed in distilled water and then immersed for 10 seconds in a 0.01% solution of potassium carbonate. The fluid was agitated by moving the slide.

4. The slide was rinsed quickly in distilled water and stained for 10–30 minutes (usually 12 minutes) with Goodpasture's acid polychrome methylene blue (1917). Good results were also obtained by staining in Masson's aniline blue (1928) for one hour. The sections were rinsed in distilled water, passed thru 95 and 100% alcohols, cleared and mounted in balsam.

In sections stained by this method, the mitochondria stained brilliant fuchsin, the eosinophilic granules orange-red, and the basophilic granules deep blue. In sections to which a less intense fuchsin stain was applied, the mitochondria took a lighter fuchsin, the eosinophilic granules orange or yellow-orange, and the basophilic granules a deep blue. The connective tissue usually stained blue while the red blood cells stained either fuchsin or orange, or intermediate between these two.

If it was found necessary, the sections intended for Golgi preparations were bleached before staining. The sections were flooded with 0.125% solution of potassium permanganate for approximately 30 seconds; they were rinsed in distilled water and were treated with a

1% solution of oxalic acid for 30 seconds. They were then rinsed in distilled water and stained by the procedures described above.

This staining procedure differs chiefly from those of Severinghaus and Volkonsky in that differentiating solutions such as picric acid and aurantia were not used to bring about the differentiation between the eosinophilic granules and mitochondria. It seems that, in tissue fixed in Champy's fluid and handled by the methods indicated above, there is a certain inherent difference between the mitochondria and the eosinophilic granules in their capacity to react to certain stains. This difference apparently makes it possible for them to be stained selectively without the use of the differentiating fluids mentioned above. The success of this procedure in differentiating the eosinophilic granules and mitochondria seems to depend on obtaining a fuchsin stain of just the optimal intensity. This depends on the heat of the hot plate at the spot where the slide is placed, the length of the heating periods and the cooling times allowed. Finally, the amount of stain applied to the slide and the area covered are probably important since these factors determine the thickness of the layer of fluid which in turn determines the rapidity with which the solution will heat. If, on the other hand, the sections are overstained in fuchsin, satisfactory differentiation between the eosinophilic granules and the mitochondria will not be obtained. We have, however, found it comparatively easy to arrive quickly at the correct intensity of the fuchsin stain.

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ROOT-TIP SMEAR METHOD FOR DIFFICULT MATERIAL

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ABSTRACT.—A technic is outlined for the preparation of difficult material for the study of chromosome number and morphology in root-tip smears. The chief objective is to obtain polar views of the metaphase plates rather than equatorial views. To achieve this end it is recommended that fresh root-tips be cut free-hand into thin cross sections. The important features are: *thin freehand cross sectioning* of the *fresh root-tips;* fixing in Belling's iron aceto-carmine solution; maceration for 2–5 minutes in 50% HCl in 95% alcohol; and mounting in "Diaphane."

The writer has from time to time successfully used several of the published root-tip smear methods. Occasionally, however, material has been encountered for which none of the ordinary methods proved satisfactory. Since the spindles of the division figures are usually oriented in the direction of the axis of the root, the smearing of either fairly large pieces of root-tip or small longitudinal strips, recommended in previous methods, results almost exclusively in equatorial views of the metaphase plate. Their success or failure depends on one's ability to diagnose such figures when flattened. Where the cell volume is large and the chromosome number relatively low this is not difficult, but in material where the cell volume is small and the chromosomes numerous, they are usually so entangled in a flattened equatorial view as to make accurate diagnosis uncertain or impossible.

To increase the proportion of polar views the following method has proven useful. After the fresh root-tips have been thoroly scrubbed free of sand and soil, they are cut into the thinnest possible cross sections, free-hand, with a sharp razor blade. With practice and care in this operation, sections will be obtained which are not more than two or three cells thick, and these are selected for mounting. In several cases this additional procedure prior to fixing has yielded results where previous methods failed (see Fig. 1). Recently, however, while endeavoring to obtain counts on tetraploid *Lactuca* species, it was found that the chromosomes were so excessively long in relation to the diameter of the nucleus that polar views could not be counted with facility. In this case the difficulty was overcome by soaking excised tips in a 0.01% solution of colchicine for 2 or 3 hours, after the method of O'Mara (1939), before sectioning and fixing.

This drug acts as a prefixation agent and greatly shortens the chromosomes increasing the clarity of the metaphase plates.

After fixing, digestion in HCl softens the material and aids in flattening. In the Feulgen technic (Heitz, 1936) a *N* aqueous solution of HCl is used for another purpose, and simultaneously works to this end. For use with aceto-carmine, a mixture of equal parts 95% alcohol and concentrated HCl (Warmke, 1935) applied for 2–5 minutes is recommended.

In flattening, care should be taken to prevent any lateral movement of the cover, as this will turn the cells on their sides and thus defeat the purpose of the method. This can usually be avoided by placing a piece of filter paper on top of the slide, and while holding it firmly in place, gently stroke it from one end of the slide to the other. After the excess aceto-carmine has been removed in this way, the slide may

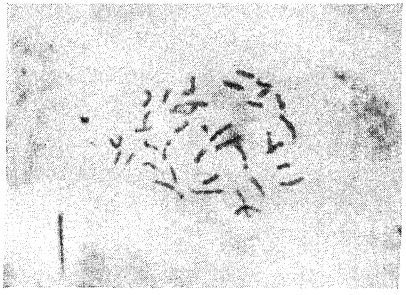

FIG. 1. Division figure from root-tip of tetraploid *Lactuca sativa* L. showing 36 chromosomes (not treated with colchicine before fixation). × 890.

be warmed slightly, and the final flattening done by pressing lightly on the cover with a needle. In making the preparation permanent, the cover is floated off in a mixture of equal parts of absolute alcohol and acetic acid, and is mounted directly in "Diaphane." This method involves the least manipulation and is therefore recommended.

The following is the complete schedule for aceto-carmine smears:

1. Where a preliminary trial indicates its necessity, washed, fresh, excised root-tips, ½ inch long, are soaked on 0.01% solution of colchicine for 2–3 hours. Otherwise omit and begin with step 2.
2. Cut fresh root-tips into the thinnest possible cross sections free-hand with razor blade.
3. With needle transfer sections to shallow vial or dish containing 2–3 drops of strong aceto-carmine. Fix for a few minutes or until very darkly stained.
4. Add several drops of 45% acetic acid to dilute the stain and

prevent overstaining. The material may be stored several days in this condition.

5. With a medicine dropper draw up a single slice and deposit on slide.

6. Holding the slide at an angle over a waste jar add, just above the slice, a drop of 50% concentrated HCl in 95% alcohol. As the first drop runs off, add more until all the aceto-carmine has been rinsed away.

7. Place the slide flat and keep material covered with additional drops of acid-alcohol for 2–5 minutes.

8. Rinse off the acid-alcohol in the same way using 95% alcohol.

9. After rinsing 2–3 minutes, dry around edges and add a drop of strong aceto-carmine.

10. After it has restained a minute or two, add a drop of 45% acetic acid and cover.

11. Blot up excess stain and flatten with heating.

12. To make permanent, float off cover in mixture of equal parts absolute alcohol and glacial acetic acid and mount in "Diaphane."

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NOTES ON TECHNIC

SUMAC WAX AS EMBEDDING MATERIAL IN BIOLOGICAL TECHNIC.[1] In an attempt to find a substitute for paraffin wax as embedding material for the preparation of microscopic slides, the wax from the seed of the lacquer tree (*Rhus sp.*) proves very satisfactory. Altho better known commercially as Japan wax, sumac wax is produced in large quantities in south-west China, being used in Kweichow, Szechuan and Yunnan for the making of soap and candles. When refined, it is a pale yellow solid with a melting point of 51° C. It is readily soluble in ether, xylol, chloroform, and castor oil; slightly soluble in warm ethyl alcohol; and insoluble in water and glycerin.

In order to test the suitability of sumac wax as an embedding material, *Lycopodium* (old strobilus), *Xylaria* (old stroma), *Auricularia*, bamboo (leaf), *Hydra*, and mouse pancreas were fixed in various fixatives (formalin, formalin-alcohol, chromo-acetic solution, or Bouin's fluid), dehydrated with alcohol, cleared in xylol or chloroform, and were finally embedded in sumac wax. The transfer of the materials from absolute alcohol to sumac wax thru chloroform may be accomplished within one day.

Sections of 1–3 μ in thickness may be made with ease on a sliding or rotary microtome. Thicker sections require careful handling due to the brittleness of the sumac wax. This brittleness is, however, to a large extent overcome by mixing 9 parts of sumac wax with 1 part of beeswax, or by mixing 3 parts of sumac wax with 7 parts of paraffin wax.

When stained with safranin and light green, safranin and gentian violet, Delafield's hematoxylin and eosin, or Haidenhain's hematoxylin, the finished preparations are in every way comparable to similar sections prepared with paraffin as the embedding material.

The only disadvantage of sumac wax compared with paraffin wax as an embedding material is its brittleness. But this may be easily removed by mixing it with a little beeswax or paraffin wax as pointed out above. The advantages of using sumac wax are: its comparatively low cost in China; the readiness with which thin sections may be obtained; the fact that it can stand a relatively high temperature (60° C.) without spoiling the material embedded in it; and the readiness with which it may be dissolved in chloroform. The fact that it is soluble in castor oil offers a further advantage to countries where xylol is more expensive than castor oil, for the latter may be employed as a clearing agent in place of xylol.—J. HSÜ and P. S. TANG, Physiological Lab., Tsing Hua Univ., Kunming, China.

[1]Aided by a grant from the British Boxer Indemnity Fund to J. Hsü.

CAUTIONS IN THE USE OF DIOXAN. Because of the frequent reference to the dioxan technic in recent papers in STAIN TECHNOLOGY, some of our readers have urged that a word of caution be included in our pages as to the possible toxic action of the chemical in question. It is generally agreed that dioxan has toxic properties; and recently Magruder[2] has thought it wise to publish a special warning on the subject, in a paper mentioning its use. He calls attention to reports that breathing only a small amount of its fumes can cause serious bronchial, liver or kidney disturbances, and states that its employment in some British laboratories has been forbidden because of its danger.

This question has been referred personally to Dr. Magruder by one of our correspondents; and in reply he states that too little experimental work has been done on the subject to justify a definite statement one way or the other as to the amount of danger involved in the use of this chemical. Presumably the danger depends on the amount of fumes inhaled; and probably there is little danger if the reagent is used in a well-ventilated room by a comparatively small number of people. When employed by a considerable number of students in a college class, Dr. Magruder recommends the following precautions: Keep dioxan containers closed (vaseline seal) when not in use. Have room well ventilated and air in circulation. Suggest that when changing tissue from one vessel to another, those containing dioxan be kept at some distance from the nose. Do not allow its use by any student who has ever had any suggestion of liver, kidney or lung trouble.

The fact that dioxan has now been employed for some time in many laboratories without any trouble seems to imply that it is not dangerous if handled with reasonable precautions. It is a valuable reagent, and should not needlessly be discarded; but the above-mentioned precautions should certainly be observed.—H. J. CONN, Geneva, N. Y.

[2]Magruder, S. R. 1938. A comparative study of dehydration. J. Lab. and Clin. Med. 23, 405–11. (See p. 411).

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

MICROSCOPE AND OTHER APPARATUS

ARDENNE, MANFRED von. **Die Keilschnittmethode, ein Weg zur Herstellung von Mikrotomschnitten mit weniger als 10^{-3} mm. Starke für elektronenmikroskopische Zwecke.** *Zts. Wis. Mikr.* **56,** 8–23. 1939.

Maximum resolution and contrast with the electronmicroscope depend upon the thinness of the sections. To obtain paraffin sections of less than 1 μ in thickness, the author designed an auxiliary tilting device to be used in connection with the usual rotary microtome which permits the cutting of "wedge" sections.

Razor blade steel has been found satisfactory. By means of a special sharpening process the facet angle can be reduced to 10°, the cutting angle to 5°, and the radius of curvature at the edge of the 5 mm. knife is 10^{-4}. The knife is held by strong jaws adjustable by set screws and put in the place of a large knife. The object is prepared as follows: Lift off one layer from object to obtain a smooth surface; replace it by a covering layer of some equally hard substance such as paraffin of the same melting point which will not alter the surface. Apply it with a fine hair brush. Tilt the object slightly. The final cut produces a wedge shaped section. Dissolve away the cover. The tissue section allows easy further sectioning. The little roll of sections is placed on a drop of water at 40° C. to flatten out, and then treated as usual.

An ordinary object holder is attached to concave surface of a Leitz-Minot microtome by means of special springs and screws allowing the slightest tilt of the object to be controlled and registered. Tilting angles of less than 0.0001 are obtainable. One turn of the screw produces a 0.02° rotation.—*E. Barasch.*

HARDING, J. P. **A simple instrument for dissecting minute organisms.** *J. Royal Micro. Soc.*, **59,** 19–25. 1939.

The paper describes the construction of an inexpensive micromanipulator based on the principle of the pantograph, and an attachment for focusing the microscope without using the hands. It is suitable for dissecting Ostracods and Copepods. The micromanipulator consists of two brass dissectors mounted on a brass base attached to the stage of a dissecting microscope. Each dissector is equipped with a manipulating knob and a dissecting needle; dissecting needles are made from .05 mm. tungsten wire by honing or by burning the tip in a small flame; dissectors are attached to the base by a ball-and-socket joint made by fitting a bicycle ball-bearing in a socket drilled in the base. Each needle is controlled by moving its manipulating knob in the required direction instead of an arrangement of mill heads. The base has a piece cut out to accommodate a depression slide. The instrument may be adapted for use with an ordinary microscope by reversing the positions of needle and ball-and-socket joint. The attachment for focusing the instrument without using the hands consists of a lever fixed to the coarse adjustment head so that it projects over the edge of the table and is approximately horizontal when the microscope is in focus. A spring or rubber band connects the lever with the back of the microscope stage and tends to raise the head of the microscope out of focus; but this is counteracted by a rod attached to the end of the lever and operated by the raising or lowering of the worker's knee. Crustacea are dissected in benzyl alcohol which is reported to be superior to glycerine for this purpose. Unstained appendages may be mounted in Hyrax (Flatters & Garnett, London) because of its high refractive index.—*H. E. Finley.*

MICROTECHNIC IN GENERAL

ENBOM, G. **Ein Schnellverfahren zur Rekonstruktion in Plastilin nebst anderen technischen Beiträgen.** *Zts. Wis. Mikr.*, **55**, 150–65. 1938.

The author proposes to substitute modeling clay for the complicated wax method of reconstruction. He indicates how casts of the temporary model can be made with cotton soaked with gelatin, and how the level of a section can be recorded with wax pencils on thin metal wires.

Slice rolls of modeling clay (Tekniska fabriken Pilen, Asarum, Sweden) into thin slices, place between linen cloths, roll out into sheets 1 cm. thick. Fold, and roll again until surface is smooth. Thin sheets out to desired thickness. Project every 2nd or 3rd section onto the clay sheets on a paper-covered projection table with a fine needle ("Cito" of K. Rasche Co., Weissbach, Germany) applied vertically. Cut out series of 10 cross sections each, completing details and outline. Stack up in order on a tray. Remove notochord and spinal cord segments, and some of "negative". Use pieces of heated iron wire, 0.5 mm. thick. Cover with celluloid sheet. Follow a profile photograph when fitting sections. Remove celluloid sheet, and the rest of "negative". Remove desired amount of body wall. Smooth out surface, remove wires.

For casts, fill in one system at a time with a thin cotton coat, pour in hot conc. soln. of gelatin appropriately colored. Let dry before starting on next system. Brush over lightly with olive oil before casting. Connect dried systems with cotton and colorless gelatin. Remove modeling mass.—*E. Barasch.*

FISHBACK, H. R. and GREGERSEN, A. **A permanent method of labelling microscope slides.** *Amer. Jour. Clin. Path., Tech. Suppl.*, **3**, 134–6. 1939.

The blank end of the slide is wiped with ether and the data written on it with India ink. This is brushed lightly and quickly with clear lacquer applied with a camel's hair brush. For contrast the back of this end of the slide is covered with white lacquer.—*George H. Chapman.*

GOMORI, G. **A differential stain for cell types in the pancreatic islets.** *Amer. J. Path.*, **15**, 497–9. 1939.

The following procedure is recommended: Fix thin slices of tissue (2 mm. or thinner) in an aq. fixative such as formalin, Bouin's, Zenker's, Stieve's, or Zenker-formalin, or better still in Bouin's fluid in which half of the acetic acid is replaced by sulfosalicylic acid and then diluted with an equal part of water before use. Embed in paraffin. Oxidize the sections after deparaffinizing with a solution containing 0.3% each of $KMnO_4$ and H_2SO_4, rinse and decolorize with a 1 to 5% solution of $K_2S_2O_5$. Wash thoroly. Stain in a well ripened solution of chrom hematoxylin made by mixing a 1% solution of hematoxylin with equal parts of a 5% solution of chromium alum, ripened 1 or 2 days after adding a few drops of H_2SO_4 to each 100 cc. (The brand of hematoxylin is not stated.) Filter before use and stain 15 min. to 1 hr. under microscopic control. Rinse and differentiate in 1% alc. HCl. Rinse and counterstain with phloxine or ponceau de xylidine (sources not given) using the latter dye as a 0.5% solution containing 1% acetic acid. Any red or orange acid dye can be used. Rinse and differentiate in a 5% solution of phosphotungstic acid until the connective tissue is decolorized. Only the strongly oxyphilic structures, such as erythrocytes, muscle fibers, oxyphils, and alpha cells, remain stained. Cover in balsam.—*H. A. Davenport.*

LEVIN, E. **Photoaufnahmen mikroskopischer Präparate ohne Apparatur sowie eine einfache und billige Methode, um Literaturbelege zu gewinnen.** *Zts. Wis. Mikr.*, **55**, 470–1. 1938.

A simple method of making photographs of low magnification is outlined by the author. According to the stain selected, simple or panchromatic plates are used. Proceed as follows: Place the coverslip of the slide on the face side of the plate, black paper under the plate to prevent halation, and expose to direct light on the ground board of the enlarging camera. For outline drawings, etc., smaller slides may be printed on normal movie film, Leica size, and the negatives enlarged. In order to retouch the negative, place it in the negative holder of the enlarging camera and make a slightly enlarged negative.

In order to make a diapositive of a freshly prepared slide use "Agfa-direct-duplicate-film" whose pre-exposure allows the second exposure to produce a

regular inversion. Printing or enlarging now produces a positive which may be projected in 1–2 hr. For the reproduction of tables or diagrams in publications, make photographs on bromide paper. Insert a clean glass plate in the camera to press it flat. Use a longer exposure and print the paper negative exactly like a film. For shaded drawings use contrast paper, and for prints extra contrast paper. Even colored and half-tone drawings may thus be photographed. This procedure saves much time and expense.—*E. Barasch.*

LIESEGANG, R. E. **Zur Theorie der histologischen Versilberung.** *Zts. Wis. Mikr.*, **55**, 415–8. 1938.

The author discusses the compounds and conditions involved in the production of nascent sliver.

Nascent silver is not colloidal since the presence of silver nuclei does not cause it to settle out. Supersaturation seems to be responsible for the formation of silver ppt., the amount varying with the concentration of the reducing agents and the purity of the medium, or presence of nuclei hastening precipitation. The following procedure may be used to study these phenomena: Make a gel from 87 cc. of a 10% gelatin solution containing 13 cc. of a 10% $FeSO_4$ solution in a beaker. Cover with 2 cc. 20% aq. solution $AgNO_3$. Metallic silver bands, some white, some black, appear in a few days. Their clearness at the bottom is enhanced by a little H_2SO_4. A smaller concentration of $FeSO_4$ alters the nature of the bands. This may be due to a precipitation of Ag_2SO_4 instead of Ag. $AgNO_3$ does not produce bands in all concentrations. After dialyzing the gelatin, in the absence of silver nuclei, an enormous supersaturation may be obtained. The distance between the colored bands is proportional to the amount of supersaturation since it indicates the migratory capacity of the silver molecule. The shade or intensity of color gives no indication as to the amount of reducing metal; e.g., the highest and lowest in a series of $FeSO_4$ concentrations produce solid coloration of the gelatin mass, intermediate solutions producing lighter bands. These may occur in any of the different forms of silver: translucent yellow or orange, solid black, dark rust, or grayish white.—*E. Barasch.*

MILOVIDOV, P. **Notizen über Mikrotechnik. III. Die Anwendung der Azetokarmin-Methode für die Färbung von fixierten Mikrotomschnitten.** *Zts. Wis. Mikr.*, **56**, 67–9. 1939.

The distribution, morphology and structure of chromosomes in root tip smears and the meiotic stages of pollen mother cells may be brought out distinctly and with good preservation by a modification of the original azocarmin method. Proceed as follows: Cover the serial sections which lie on the slide, free from paraffin and washed with water, with a few drops of Schneider's azocarmin solution. Warm slightly for 1–2 min. over micro-burner. Cool, wash with water, transfer for a few minutes to 96% alcohol, then successively to abs. alcohol, xylol alcohol, and pure xylol. Embed immediately in Canada balsam. Do not use much albumen in attaching the section to the slide. The procedure is rapid, avoids artifacts, aids in the study of meioses, and even stains cell membranes well.—*E. Barasch.*

PITOT, A. **Une méthode rapide d'exploration des lames.** *Bull. d'Histol. Appl.* **15**, 77–8. 1938.

To avoid waste of time by mounting unnecessary sections, they may be solidified in Canada balsam and examined before mounting. To do this, after removing sections from the paraffin bath, cover with a thin coat of liquid Canada balsam (75 g. of dry, pulverized balsam dissolved slowly in 100 cc. of xylene). After 2 or 3 min. drain off excess liquid. Place on filter paper, and dry 2 to 4 hr.

The sections can be examined under fairly high magnification, and can be kept some time. Cracked and dust-covered sections may be repaired by dissolving in xylene and refreezing.—*Jean E. Conn.*

POLICARD, A. **La Methode de la Microincineration, Exposé Practique.** 6½ by 10 in., 50 pp. Paper. Herman et Cie., Paris. 11 francs.

The author of this well written and interesting booklet, by his many worthwhile studies on mineral distribution in cells and tissues, has in recent years, renewed interest in a field of investigation which had been dormant since the work of MacCallum at the beginning of the century. There is little doubt that micro-

incineration, if properly performed with tissues prepared by the frozen dehydration method, gives a reasonably true representation of mineral distribution in living cells and organisms. Furthermore, it is the only method extant which permits the appreciation of total mineral dispersion in biological materials. Altho the interpretation of such findings and their importance in physiology is difficult at present, they have given several new concepts of cellular organization in health and disease.

Professor Policard's monograph proceeds in an orderly and direct fashion from an historical resumé as introduction to the table of contents which takes the place of an index. The paragraphs dealing with fixation of tissues are perhaps too few, and possibly the frozen dehydration method could have been profitably included (instead of dismissing in a paragraph); but the author is not to be criticized severely in this instance since he is presumably writing primarily for readers not deeply concerned with intracellular localization of minerals.

Details of the technical procedures of incineration are dealt with carefully and thoughtfully. Many pitfalls in the technic are pointed out and artifacts and their causes described. Particular attention is given to the methods of examination of the preparations. The difficult question of interpretation of the incinerated tissue sections is handled cautiously and with no apparent evidence of wishful thinking.

Especially valuable is the third section of the booklet which is devoted to "Techniques complémentaires", for here the many accessory methods devised by workers in the field are described and discussed. The paragraphs devoted to chemical reactions, microphotometry and allied methods are well done in most respects. The bibliography is carefully selected and contains the titles of review articles on the subject.

Professor Policard's booklet should be of great value to the beginner who hopes to apply the method to biological problems. Perhaps one of its greatest uses is in bringing together the many laboratory artifices which have grown up around the basic technic.—*Gordon H. Scott.*

SCHENKEN, J. R., and McCORD, W. M. **A method for the examination of the cellular elements of body fluids. A collodion sac for concentration and paraffin-embedding.** *Amer. J. Clin. Path., Tech. Suppl.* **3**, 176–8. 1939.

Fill a 15 cc. chemically clean, dry centrifuge tube with fresh collodion, U.S.P. Pour out the collodion with a rotary motion so that the lip of the tube is uniformly and completely covered. Clamp the tube in an inverted position and drain 10 min. Rinse with water and separate the sac with the aid of a stream of water from a wash bottle. Test sac for leaks by filling it with water. Store it in 1:10,000 merthiolate. When ready for use, empty sac and fill it with fluid to be tested. Put a thin, moist glass rod in a 15 cc. centrifuge tube moistened with saline and use the rod to help in inserting the filled sac. Withdraw the glass rod. Fill the space around the sac with the fluid to be examined, or with saline. Fold the edge of the sac over the edge of the tube and secure it firmly by a cork, taking care to exclude air from the sac. Centrifuge. Remove sac and tie it off with No. 20 thread about 3 mm. above the sediment. Cut off the empty part of the sac and discard it. The tied portion may be fixed, dehydrated and embedded in paraffin by usual technics except that acetone and other collodion solvents cannot be used.—*George H. Chapman.*

SYLVÉN, B. **Experimentelle Beiträge zur Kenntnis der Färbung mit Mucikarmin nach P. Mayer.** *Zts. Wis. Mikr.*, **55**, 462–9. 1938.

The author studies first the effect of H-ion concentration on solutions of mucicarmine, or aluminum carminate using the following procedure: Prepare 1% solutions of mucicarmine, diluted with 10 parts of dist. water and a series of solutions of HCl ranging from pH 6.0 to pH 1.0. After 24 hr. the solutions with pH values above 3–4 appear clear and violet; those below appear red with a brownish flocculent ppt. The red color is due to acid carmine. Add a few drops of conc. NH_4OH to a 0.1% solution of mucicarmine. A thick violet red gelatinous ppt. forms, soluble in excess alkali. It consists of $Al(OH)_3$. Tap water dilutions from 1/400 to 1/60 produce the same result in 3–12 hr.; with dist. water, the dilutions last 6 days or more.

Next, the staining effect of mucicarmine solutions of different acidities is studied. Staining period 1 hr., pH 2–7; for acid carmine, 20 hr. The staining

capacity of mucicarmine lies between pH 4–7. A neutral solution produces diffuse red coloration, and at pH 2 the acid carmine stain takes place. Since chondroitin-H_2SO_4, as in cartilage, and mucoitin-H_2SO_4 are alkali soluble, lower acidities cannot be applied. Free radicals of these compounds may be bound to one or more of the trivalent Al-ions of Aluminum carminate.—*E. Barasch.*

WOLF, JAN. **Die Glyzerinmethode der Zelloidineinbettung.** *Zts. Wis. Mikr.*, **56**, 57–62. 1939.

This method was devised to shorten the time required for the celloidin technic. Reagents required: Glycerin (ordinary); Glycerin 31 Bé (100% glycerin is 30 Bé. *J. M. T.*); 8% celloidin in abs. alc. and ether. The fixed and washed material, (e.g. a piece of liver 3×6×6 mm.) is placed directly into ordinary glycerin for 6 hr.; from this it is transferred to anhyd. glycerin 31 Bé for an additional 6 hr. The bottle containing the glycerin and specimen should be turned upside down frequently to insure thoro infiltration.

The usual amount of 8% celloidin required for infiltrating and blocking the specimen is placed in a suitable container and the block of tissue, wiped free of excess glycerin, placed therein. Infiltration and inspissation of the celloidin is complete in 24 hr. The block can be mounted before transferring to 80% alcohol for hardening and preservation.—*J. M. Thuringer.*

DYES AND THEIR BIOLOGICAL USES

BUGYI, B. **Reinheitsprüfung der histologischen Farbstoffe.** *Zts. Wis. Mikr.*, **55**, 198–210. 1938.

An easy method of testing for impurities in histological dyes by means of capillarity is described, and the results are presented for 38 different dyes. The only objectionable impurities are the diffusible substances which alter the color of the dye.

Cut strips of plain filter paper 2 cm. wide, 30 cm. long. Suspend 24 hr. in staining dish (10 cm. deep, 3–5 cm. wide) containing 15–20 cc. 1% solutions of the dye in dist. water, Ringer's solution, alcoholic, acid, and basic solutions.

Azocarmine seems to contain impurities. The impurity found with brilliant black is more diffusible than the dye (color, brown). Carmine was found pure, but acid carmine impure. Cresyl violet contains slight impurities. Dahlia is a compound dye producing a narrow red zone at the top of the diffusion picture. Ringer's and alc. solutions bring out impurities in erythrosin. A pink zone appears in a basic solution of methylene blue, due to the partially demethylated product. There is a violet impurity found in malachite green. Metanil yellow is not quite pure. Violet tinges are found with methyl green, probably due to parafuchsin derivatives. Nile blue sulfate is not a pure dye. Orange G seems impure; so does orcein D. Safranin may contain an impurity. There are red and blue zones in thionin. There is a red impurity in trypan blue.

Nearly all metachromatic dyes show impurities, but in general the purity of dyes has been greatly improved; dextrin, in fact, is almost never present.—*E. Barasch.*

FIGGE, F. H. J. **Melanin: a natural reversible oxidation-reduction system and indicator.** *Proc. Soc. Exp. Biol. & Med.*, **41**, 127. 1939.

Melanin was found by the author to be a natural redox system comparable to glutathione, riboflavine and cevitamic acid. The reduced form decolorized toluylene blue and other indicators; the oxidized form oxidized reduced indigo disulphonate, methylene blue and thionine. Oxidized melanin absorbed 70% of light (water given 0.0 absorption value); reduced with Na hydrosulfite only 25% was absorbed; reoxidized, 70% was again absorbed.—*M. S. Marshall.*

LEVINE, M. **Crown gall-like tumors induced with scharlach red on the plant, Kalanchoe.** *Proc. Soc. Exp. Biol. & Med.*, **40**, 599–603. 1939.

Crown-gall-like overgrowths, characterized by leafy shoots and roots, were produced on decapitated shoots of *Kalanchoe Daigremontiana* by scharlach red dissolved in ether. Other carcinogenic agents, as 1,2,5,6-dibenzanthracene, methylcholanthrene and benzopyrene, applied in lanolin caused injuries without inducing overgrowths. Stimulation of root formation was observed with some substances, including indole acetic acid and lanolin used alone.—*M. S. Marshall.*

STOW, W. P. **Rose bengal excretion and hippuric acid synthesis tests of liver function: a comparison.** *J. Lab. & Clin. Med.*, 24, 866–8. 1939.

The author compares the results obtained with Delprat and Stowe's rose bengal excretion test and Quick's hippuric acid synthesis test in seven patients with liver dysfunction. Striking agreement is evident. It is proposed that the rose bengal test be used where speed is important, requiring only 1 hr. as compared to 12 hr. or more for the hippuric acid test. Quick's test is recommended where severe jaundice is present and in laboratories with limited reagents and apparatus. —*Parker R. Beamer.*

ANIMAL MICROTECHNIC

AURELL, G. **Kolophonium-Chininhydrochloridgemische als Einschlussmittel für sehr dicke Schnitte zu microskopischen Zwecken.** *Zts. Wis. Mikr.*, 55, 256–73. 1938.

The purpose of this study is the introduction of Spalteholz' clearing method to micro-anatomy, supplementing present-day methods of reconstruction and replacing them in some cases.

The main requirement is a suitable embedding medium which is pure and does not crystallize and which has a refractive index similar to that of the tissues. To determine the refractive index of an organ, place the material in mixtures of different proportions of benzyl benzoate (r = 1.568) and methyl salicylate (r = 1.536). The combined index should fall somewhere between 1.560 and 1.550. Compare transparence of embedded sections by looking for cell walls. At the optimum refractive index they are invisible.

Proceed as follows: inject material for blood vessels, or stain with any dye except picric acid. Bleach 1 mm. sections of different organs with H_2O_2. Dehydrate completely in alcohol. Place sections in benzol. Transfer to clearing mixture, 12–24 hr. For microscopic study, embed material using anise oil (r = 1.557) as a solvent and colophonium with quinine-HCl as a medium. Dissolve quinine-HCl with gentle heating in anise oil containing measured amounts of colophonium. Let cool completely; add remainder of resin. Let stand for a few days. Place dry tissue into anise oil, then in medium. Leave it there one day. Section with freezing microtome. Study with stereoscopic microscope.—*E. Barasch.*

HOLMGREN, H. **Eine neue Methode zur Fixierung der Ehrlichschen Mastzellen. Mit besonderer Berücksichtigung der Chemie der Zellgranula.** *Zts. Wis. Mikr.*, 55, 419–61. 1938.

Mast cells produce a secretion similar to heparin in its clotting effect and consisting of more or less water-soluble polyester sulfuric acid granules. This fact is the basis for the author's use of fixatives known to precipitate such compounds. Proceed as follows: Fix organs, such as liver, lung, kidney, spleen or ovary of various vertebrates, with a fresh basic 4% aq. solution of PbAc in a well closed bottle, 12–24 hr. Wash with dist. water. Transfer to 60% alcohol. Dehydrate, embed in paraffin. Between fixation and dehydration a second series may be washed for 24 hr., and a third series treated 24 hr. with a neutral solution of 10% formalin. To compare with the PbAc method, use for fixation: 1) quinine-HCl (4%) in conc. aq. solution and in 25, 12.5, and 5% alc. solution; 2) conc. aq. solution of $Ba(OH)_2$, and $BaCl_2$; 3) neutral solution of formalin (10%) followed by alcohol treatment or by washing for 24 hr. with water, or fixing in formol sublimate for 12 hr.; 4) Helly's solution; 5) Maximov's solution. Section blocks and stain, using toluidine blue, 1) in 1% aq. solution; 2) in 60% (1 g. in 99 cc.) alc. solution, which gives a specific metachromatic reaction with high molecular ester-H_2SO_4 compounds.

A heparin injection produces granules similar to those in Ehrlich's mast cells in endothelium which stain best with PbAc fixation. At a certain concentration of alc. solution (90%) of toluidine, high molecular carbohydrates with few ester groups no longer stain, whereas the polyester granules retain their staining property.—*E. Barasch.*

LANDAU, E. **Contribution aux methodes myeliniques.** *Bull. d'Histol. Appl.* 15, 181–5. 1938.

The author has refined his earlier technic for staining myelin sheaths so that it gives good results for small pieces as well as hemispheres and whole brains. The procedure is as follows: (1) Fix 10–14 days in 10% formol. (2) Rinse 24 hr. in

running water. (3) Fix 24 hr. at 25–30° C. in the following solution: $K_2Cr_2O_7$, 5 g.; 10% aq. $FeCl_3$ or iron alum, 20 drops; dist. water, 100 cc. (4) Wash in running water 6–12 hr., according to the size of the piece. (5) Dehydrate successively. (6) Abs. alc. (7) Chloroform, change 2 or 3 times. (8) Embed in paraffin. (9) Section at 15μ. (10) Attach the sections with gelatin or dist. water. (11) Remove the paraffin and mordant 3–6 hr. at 25–30° C. in a solution of 10% $FeCl_3$ or 10% iron alum. (12) Rinse 2 sec. in dist. water. (13) Stain several hours in 1% aq. hematoxylin at 25–30° C. (14) Wash 1–2 hr. in running water. (15) Check the differentiation in K ferrocyanide from time to time under the microscope (K ferrocyanide 2.5 g. and sat. aq. soln. Li_2CO_3, 100 cc.). (16) Wash several hours in running water. (17) Counterstain, if desired, with Hollburn's "Kernechtrot" (Kernechtrot, 0.1 g.; $Al_2(SO_4)_3$, 5.0 g.; dist. water, 100 cc.) 5 min. (Domagk's mixture). (18) Dehydrate, mount in balsam.—*Jean E. Conn.*

LILLIE, R. D. **Experiments on the solubility of hemosiderin in acids and other reagents during and after various fixations.** *Amer. J. Path.*, **15**, 225–39. 1939.

The acid solubility of iron-containing pigment in sectioned tissues from man and laboratory animals was studied after treatment with a number of commonly used fixatives. The state of the Fe in hemosiderin was shown to be ferric. Fixation in acid fixatives which contained no formalin either removed hemosiderin or extracted the iron from it, leaving a non-ferrous brown pigment. After fixation in formalin or in formalin-containing fixatives, hemosiderin became more resistant to subsequent extraction by acids. In general, the efficacy of various acids used in aq. solutions to extract hemosiderin after formalin were, in order of decreasing efficacy: oxalic, sulfuric, nitric, formic, and hydrochloric. There was considerable variability in the time required for removal of the pigment. Four hours in 10% H_2SO_4 (aq.) at 25° C. was sufficient in many preparations but some required either higher temp. (37°) or a longer treatment. The age of the pigment had little influence on its speed of solution, but that which had coarse granules resisted solution longer. The iron-free pigment, hemofuchsin, was sometimes present after acid-containing fixatives or after treatment of fixed tissue with warm acids. This pigment was believed to be derived from hemosiderin by loss of iron. The evidence indicated that the iron existed originally in combination with protein.—*H. A. Davenport.*

LADEWIG, P. **Über eine einfache und vielseitige Bindegewebsfärbung. (Modifikation der Mallory-Heidenhainschen Methode).** *Zts. Wis. Mikr.*, **55**, 215–7. 1938.

A simple method for staining connective tissue elements evenly and constantly without precipitation is described by the author. The procedure is as follows: fix material, preferably in formalin. Make paraffin sections 5–10 μ thick. Place in xylol, and run thru decreasing concentrations of alcohol to water. Place in Weigert's iron hematoxylin, 3–5 min. Wash briefly with dist. water. Transfer sections to 5% phosphotungstic acid, 1–3 min. This stains the connective tissue elements light red, the nuclei remaining dark brown. Wash briefly with dist. water. Place for 4 min. in staining fluid (1 g. acid fuchsin, added to 100 cc. of Mallory's original solution, boiled briefly and filtered when cold). Wash briefly with dist. water. Transfer directly to 96% alcohol, 1 min., moving sections slightly. Place in abs. alcohol, xylol, and balsam.

Results: nuclei, dark brown; connective tissue, from blue to carmine; parenchyma, gray blue or brown; nervous tissue, the same; muscle, reddish brown; erythrocytes, light orange; fibrin, bright red; mucus and secretions, blue; amyloid, glassy light blue; colloid, violet to red; calcium, red; keratin, red; eleidin, orange; transitional tissue, violet; keratohyalin, dark brown.—*E. Barasch.*

LADEWIG, P., and DESSAU, F. **Ergebnisse mit einer neuen Bindegewebsfärbung.** *Zts. Wis. Mikr.*, **55**, 211–4. 1938.

The authors have investigated Ladewig's modification of Mallory's connective tissue stain using the "azan" procedure for control sections. Collagenous fiber tracts, in vascular walls, in scar tissue, in inflammatory changes, and in some tumors, stain red, violet or blue, the same shades appearing in similar sections which all indicate some fibrinoid proliferation in a broad sense. Fuchsinophil connective tissue indicates injury to the tissue, but some injuries are not thus represented. The distinctness with which fibrin and paraplastic substances stain

makes the procedure useful for the diagnosis of necrosis and growth processes. Since erythrocytes stain yellow to orange, i.e., differently from all other elements, the slightest hemorrhages can thus be detected. Amorphous calcium and bone trabeculae appear bright red, while osteoid tissue is bluish. Changes in ovary and uterus which take place mostly in the connective tissue elements may be easily followed. Parenchymatous organs and epithelial structures are not stained intensively enough to obscure the finer details of cell structure. Main and parietal cells in the gastric fundus are easily distinguished, as are also the three types of cellular components of the hypophysis, and keratin, eleidin, and keratohyalin.—*E. Barasch.*

MOLLIER, G. **Eine Vierfachfärbung zur Darstellung glatter und quergestreifter Muskulatur und ihrer Beziehung zum Bindegewebe.** *Zts. Wis. Mikr.*, **55**, 472–3. 1938.

Since azocarmin does not stain myofibrils and nuclear structure in sufficient detail, the author proposes the following modification of the "azan" staining method: Transfer the sections from 10% alcohol to orcein (12 hr.). Wash well with dist. water until no more color comes off, and place in Weigert's hematoxylin for 1–3 min. Wash with dist. water. Differentiate briefly using 0.1% HCl in 70% alcohol until nuclei are distinct. Wash 15 min. in running tap water. Stain 15–30 min. with cold azocarmin G. Wash with dist. water. Place 3–6 hr. in 5% phosphotungstic acid, changed 2–3 times until collagenous fibers are completely decolorized. Wash with dist. water. Stain 15–30 min. with naphthol green B (1 g. in 100 cc. dist. water, 1 cc. glacial acetic acid). Transfer directly to 96% alcohol for 30 sec., stirring to prevent precipitation of green. Pass thru alcohol, xylol, balsam. Result: chromatin, nucleoli, deep blue; cytoplasm, bluish-red; myofibrils, deep red with sharp contours; elastic fibers, dark brown; collagenous fibers, olive; erythrocytes, bright red. Start with Weigert's stain, if elastic stain is to be omitted. This stain is prepared as follows: Solution I: dissolve 1 g. hematoxylin in 100 cc. 96% alcohol, add 0.2 g. $NaIO_3$ and 62 cc. dist. water. Solution II: 4 cc. liquid ferrous bichlorate, 96 cc. dist. water, 1 cc. officinal HCl. Immediately before use 1.5 parts of solution I and 1 part of solution II are poured together and mixed. Do not use mixture longer than 2 hr. —*E. Barasch.*

MONNE, L. **Über Vitalfärbung tierischer Zellen mit Rhodaminen.** *Zts. Wis. Mikr.*, **55**, 143–9. 1938.

The author presents the results of his vital staining experiments with electrically neutral rhodamine B, Schultze (1923) No. 573. When injected into amebae, the ground cytoplasm is stained diffusely. In other cells, e.g., spermatocytes and spermatids of *Helix*, oocytes of *Helix* and *Arachnida*, frog muscles and *Opalina ranarum*, the dye stains the ground cytoplasm diffusely, the mitochondria more intensely.

Between crossed Nicol prisms of the polariscope cells stained with rhodamine B and 6G (Schultz No. 571)—which stain alike—show an orange-red fluorescence. Oocytes and embryos of the *Ascidia*, *Styela partita*, contain differently staining primary tissues: blastomeres forming ectoderm and muscles do not stain; those giving rise to the intestine stain intensively; and those forming the notochord, weakly. The circumnuclear plasma of the entomeres does not stain.—*E. Barasch.*

PRICKETT, C. O. and STEVENS, CORNELIA. **The polarized light method for the study of myelin degeneration as compared with the Marchi and Sudan III methods.** *Amer. J. Path.*, **15**, 241–50. 1939.

The lumbosacral and sciatic nerve trunks from 142 rats were studied as follows: 71 were used as controls, the remaining 71 had the right nerves cut and allowed to degenerate for periods of 24, 48, 72, 120 and 216 hr. respectively in different groups of animals. For study by polarized light and Sudan III, the tissues were fixed in neutral formalin (Merck); for Marchi, in Müller's fluid. Frozen sections were used for polarized light and Sudan III. The polarized light method was found to be rapid and accurate; the certainty of recognizing changes characteristic of degeneration was present 24 hr. after transection. The earliest degeneration shown by the Marchi method was 72 hr., and by Sudan III, 120 hr. after transection. The Marchi method was inconsistent as compared with results obtained by

polarized light. The Sudan III method was consistent but failed to show the earlier changes. The observations of Setterfield, Sutton, and Baird (Stain Techn. **11**, 41. 1936), relative to the superiority of polarized light over Marchi's staining in the detection of degenerating myelin sheaths of peripheral nerves, were confirmed.—*H. A. Davenport.*

ROSKIN, G. **Sur la diagnostic cytologique différentiel des cellules cancéreuses.** *Bull. d'Histol. Appl.* **15**, 20–3. 1938.

Staining with the leucobase of methylene blue (Rongalitweiss of Unna) leaves cells from malignant tissues colorless, but normal tissues are stained an intense blue. This was tested on tumors of the white mouse, rat, chicken, guinea pig, and man.

To prepare the "rongalitweiss", use a 5% solution of methylene blue (to which 7 drops of 25% HCl have been added). To 10 cc. of this solution add 0.3 g. of rongalite (of which the quality is important). Heat carefully until colorless or pale yellow. The pH should not be lower than 2.41. Use tissues dried in the air. Stain 2 min. Wash quickly but carefully in O-free water (kept free of O by a thin coat of liquid paraffin). Dry on filter paper and seal with paraffin.

Normal tissue can even be distinguished macroscopically from malignant tissue by the difference in color. This method is suggested as a supplement to present methods of diagnosis.—*Jean E. Conn.*

TURNER, OSCAR A. **A manual of neurohistologic technique.** *J. Lab. & Clin. Med..*, **24**, 735–48. 1939.

This is the first of several installments dealing with general considerations, stains for nerve cells, myelin sheaths, neurofibrils, fats, glia, connective tissue, pituitary and pineal body, spirochetes and miscellaneous methods. One chapter near the end of the article is to deal with formulas and the entire article is to be supplemented with a list of selected references.

In this installment fixation of tissues, removal of tissue, laboratory equipment and gelatin embedding are discussed. Procedures for the following staining methods are given:

(1) For staining myelin sheaths: Pal-Weigert, Spielmeyer, Weil rapid method, Wolter-Kulschitzky, and the Marchi method for degenerating myelin.

(2) For staining neurofibrils: Bielschowsky's silver method and Cajal's method.

Certain advantages and disadvantages of each method are also mentioned together with hints as to the technics of performing them.—*Parker R. Beamer.*

TURNER, OSCAR A. **A manual for neurohistologic technique.** *J. Lab. & Clin. Med.*, **24**, 871–87. 1939. (Second installment in series)

Complete procedures and comments are given regarding the following stains:

(1) For fats. Sudan III, Herxheimer's method (utilizing a derivative of Sudan III) and Nile blue sulfate.

(2) For staining interstitial cells, a division into metallic and non-metallic stains has been made for convenience. *Non-metallic methods*: Mallory's phosphotungstic-acid-hematoxylin stain, Bailey's ethyl-violet-orange-G stain for fibrillary neuroglia, Holzer's glia-fiber stain, and Alzheimer-Mann's method for neuroglia. *Metallic methods*: Cajal's gold sublimate method and Corten's modification, Kanzler's modification of Hortega's method for microglia, Penfield's second modification of Hortega's silver carbonate method, Wilder's method for glia and nerve fibers and Fincher's silver carbonate method.

Glia stains on paraffin sections are mentioned briefly with the comment that they usually are not very reliable and rarely are they selective.—*Parker R. Beamer.*

WEISSCHEDEL, E. and JUNG, R. **Die anatomische Auswertung und das Studium der secundären Faserdegeneration nach lokalisierter subcorticaler Ausschaltung durch Elektrokoagulation.** *Zts. Anat. u. Entwgesch.*, **109**, 374–95. 1939.

The staining of degenerating myelin is discussed in the light of carefully controlled experiments with the tract degeneration in the central nervous systems of cats and rabbits. The original Marchi method is compared with the chlorate-osmic-formalin method (Swank and Davenport, Stain Techn., **10**, 87–90. 1935.) The latter method is considered to have advantages over the original Marchi by

requiring less osmic acid, by saving time, and by giving a clearer background. It was found to be less suited for beginners because of lack of uniform impregnation. Counterstaining with cresyl violet from Bender and Holbein after bleaching sections with $KMnO_3$ and oxalic acid was best. Authors call for differentiation of cresyl violet in 1% alc. HCl. (This is apparently an erroneous quotation from Swank and Davenport and should be 0.01%.) They recommend alum-cochineal for counterstaining after the regular Marchi stain. A rapid embedding method is given which employs 1 part anhyd. $CuSO_4$ in 4 parts acetone for dehydration; followed by 3 to 5 changes of abs. alc., at 2 to 3 min. intervals with shaking; alcohol-ether 1 to 2 min.; 2 to 3% celloidin, two changes of 5 min. and 10 min. respectively, during which time the 4 to 6 mm. slices of tissue must be turned carefully and the fluid kept agitated. The individual slices of the original piece of tissue are then built up on a previously prepared celloidin base 3 to 5 mm. thick into the composite block, the whole immersed in thick celloidin and hardened in chloroform. The solvent action of alcohol-ether and celloidin solutions on degenerating myelin is thereby avoided.—*H. A. Davenport.*

ZACH, O. **Die naturgemässe Zelloidin-Einbettung.** *Zts. Wis. Mikr.*, 55, 299–307. 1938.

The author describes the disadvantages of the common celloidin embedding method and proposes the following process for improvement of irregular hardening and tearing: Soak the material well in celloidin, as usual. Place it in an embedding vessel containing 8% celloidin solution. Spread well. For inspissation place material into desiccator containing a spoonful of $CaCl_2$. No lid is needed. Open desiccator every 12 hr. Remove contents, aerate well, replace contents. The $CaCl_2$ also prevents much evaporation of alcohol. In 2–3 days the celloidin sets in a soft gel covered by a tougher skin. Free it from the edges with a small knife. When only traces of ether remain, take out the $CaCl_2$ every 12 hr. and burn it. Replace the dry salt. In a few days the celloidin may reach the desired hardness. Free it from embedding vessel. If bottom is still soft, wait 1–2 days more. For some kinds of tissue, the block may have to be dried further. Place blocks in 90% alcohol containing a little safranin, 24 hr. Pour off the alcohol. Place in an alcohol-turpineol mixture, 3:1, 24 hr., alcohol-turpineol, 1:1, 24 hr., turpineol, several days. Add a little safranin to mixtures, preventing decolorization. Change turpineol until alcohol from blocks no longer reddens it. The blocks may be sectioned a few days later with a dry knife, cutting at a 45° angle.

It is easy to cut 14 μ sections. With care, they may be made 10 μ thick; 6 μ sections are more difficult, but even 4 μ sections may be made of brain and gland tissue.—*E. Barasch.*

PLANT MICHROTECHNIC

KOONZ, C. H. and RAMSBOTTOM, J. M. **A method for studying the histological structure of frozen fruits. 1. Poultry.** *Food Res.*, 4, 117–28. 1939.

A Pyrex desiccator containing P_2O_5 serves as the drying chamber. The parts are sealed with vaseline diluted with kerosene. It is connected thru two dry ice traps to a Cenco-Hyvac pump operating with a light oil. Samples of the frozen poultry are cut several millimeters in diameter and about 1 cm. long. After 24 hr. the desiccator stopcock is closed, the pump stopped and the desiccator allowed to warm up to room temp. The dried and fixed tissues are taken out and immediately plunged into melted paraffin. (If taken out while extremely cold they will absorb considerable moisture from the air.) If properly dehydrated the tissues will sink to the bottom of the paraffin within a few minutes. Sections should not be floated on water because this tends to produce coarse breaks. Sections are preferably placed on dry slides.—*George H. Chapman.*

MICROÖRGANISMS

GIEMSA, G. **Einfache Dauerfärbung von Hautpilzen und Mikroorganismen mit Azureosinglycerin. Bemerkungen zur Arbeit von Erich Hoffmann, diese Wschr. 1938, 1622.** *Klin. Woch.*, 18, 133–4. 1939.

The Giemsa stains give quite different results in tissue staining depending on whether or not they are used in fluids in which they dissociate. The author

observed and reported this phenomenon many years before E. Hoffmann was puzzled by the apparent reversal in staining reaction.—*V. W. Kavanagh.*

HEWITT, REDGINAL. **A staining technic for demonstrating avian malaria parasites in tissue sections.** *Amer. J. Hyg.* **29,** *Section C,* 115–7. 1939.

A modification of the Wollbach's Giemsa stain is given. After fixation in Zenker formol and imbedding in paraffin, sections of liver, spleen and bone marrow are cut 5–10μ thick. The sections are mordanted for 30–60 min. in 2.5% $K_2Cr_2O_7$, then stained 24 hr. in the following solution: dist. water, 100 cc.; 0.5% $NaHCO_3$, 2–4 drops; methyl alcohol, 3 cc.; Giemsa's stain (Azure I eosin, National Aniline, 3 g.; Azure II, 0.8 g.; methyl alcohol, 250 cc.; glycerol 200 cc.) 2.5 cc. Differentiate in 70% alcohol and dehydrate in xylol-acetone mixtures. Mount in neutral balsam or cedar oil. This technic gives good results in staining tissues of canaries infected with *Plasmodia cathemarium.* Mature erythrocytes stain orange and the parasites within them stain blue. The chromatin of the parasite does not stain red as in blood films.—*John T. Myers.*

KUROTCHKIN, T. J. **The value of Gram's stain for differentiation of pathogenic fungi.** *Chinese Med. J.*, Suppl. **2,** 337–41. 1938.

One hundred twenty species of all known groups of pathogenic fungi were stained by Gram's method, employing 0.02% aq. safranin for counterstain. Four groups were differentiated by staining: (1) Fungi completely Gram-negative, e.g. *Trichosporon*; (2) Fungi showing Gram-negative mycelia and Gram-positive spores, e.g. *Penicillium, Aspergillus*; (3) Fungi completely Gram-positive, e.g. *Trichophyton*, and *Microsporon*; (4) Fungi partially Gram-positive, e.g. *Sporotrichum.* References are given.—*Sara A. Scudder.*

MORTON, H. E. **The survival of microorganisms in fixed and stained preparations.** *Amer. J. Clin. Path., Tech. Suppl.*, **3,** 68–70. 1939.

Among about 40 preparations tested by flaming three times and then testing for viability in infusion broth, *Staphylococcus aureus, S. albus,* Pneumococcus Type I, *Streptococcus faecalis* and *Mycobacterium phlei* survived. Two cultures survived 1 min. in basic fuchsin; one in Hucker's gentian violet 1 min.; four in aqueous safranin 1 min.; and two in Loeffler's alkaline methylene blue 15 min. These results indicate that care should be taken in handling such preparations.—*George H. Chapman.*

OGDEN, M. A. **A new method of making smears for hematologic, cytologic and bacteriologic examinations.** *Amer. J. Clin. Path., Tech. Suppl.*, **3,** 183. 1939.

Put one drop of test fluid near the edge of a slide. Hold at an angle of 35° and smear on a second slide from the center to the end. Place 3–5 drops on the other half of the slide, and spread, making a thick smear. This provides a thick and a thin smear on the same slide. If cells are numerous the thin half can be examined. If cells are scarce the thick half will be more suitable.—*George H. Chapman.*

v. HORVATH, J. **Eine neue Silbermethode für die Darstellung der erregungsleitenden Elemente der Ciliaten.** *Zts. Wis. Mikr.*, **55,** 113–22. 1938.

The purpose of this method is the representation of the nervous structures of ciliates, independent of sun or artificial light exposure, using the reduction of formalin by NaOH. The procedure is as follows: fix 2–60 sec. with concentrated formalin, (diluted 3–4 times with culture fluid—i.e. approximately 10% formalin). Centrifuge and pour off solution without washing. Place 3–4 min. in 1–2% or 10% aq. solution $AgNO_3$. Centrifuge, pour off $AgNO_3$ solution without washing. Place in 1–3% aq. solution NaOH. A brown or black ppt. is formed; that previously formed by formalin is dissolved. Centrifuge material briefly at high speed. Wash in series of alcohols, then in alc. gylcerol; if alcoholic material turns gray under cover glass, omit alcohol and use dilute gylcerol.

This method is not specific for nervous elements as it also stains the supporting network of ciliates. It may be applied to the study of higher organisms.—*E. Barasch.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the July number of this Journal.

STAINS CERTIFIED JUNE 1, 1939 TO AUG. 31, 1939*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Janus green B	NJ–7	58%	As vital blood stain	June 5, 1939
Erythrosine	NEr–5	88%	As histological counter-stain	June 5, 1939
Nile Blue A	LNb–2	76%	As stain for fats and fatty acids	June 6, 1939
Malachite green	CMg–4	98%	As histological and bacteriological stain	June 19, 1939
Wright Stain	CWr–12	....	As blood stain	June 23, 1939
Malachite green	LMg–4	96%	As histological and bacteriological stain	June 26, 1939
Methylene blue	LA–8	85%	As histological and bacteriological stain and as constituent of blood stains	June 26, 1939
Pyronin G	NP–7	55%	As constituent of Pappenheim's stain	June 26, 1939
Sudan III	NY–6	90%	As fat stain	July 18, 1939
Hematoxylin	FH–15	....	As histological and cytological stain	July 18, 1939
Aniline blue WS	NK–4	....	As histological stain	Aug. 7, 1939
Brilliant cresyl blue	NV–16	60%	For vital staining of blood	Aug. 7, 1939
Eosin Y	NE–14	87%	As histological stain and as constituent of blood stains	Aug. 16, 1939
Crystal violet	NC–20	89%	As histological, cytological and bacteriological stain, and in bacteriological media	Aug. 22, 1939
Malachite green	NMg–7	94%	As histological and bacteriological stain	Aug. 23, 1939

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

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